Tissue tropism and non-lethal detection of *Nucleospora cyclopteri* (Microsporidia) in lumpfish (*Cyclopterus lumpus* L.)

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Abstract

In the present study, wild-caught mature lumpfish (*Cyclopterus lumpus*) from a brood fish fishery in Norway were examined for the microsporidian parasite *Nucleospora cyclopteri.* This was done in order to reveal the tissue tropism of the parasite and to establish non-lethal sampling methods for detection. This effort will be important for establishing *N. cyclopteri*-free brood stock. The parasite may be vertically transmitted and have immunosuppressive effects on the host and could be important for reducing the mortalities of lumpfish in sea pens. This is essential for optimizing the use of cleaner fish with salmon and thus reducing the numbers of salmon louse in the sea.

Nucleospora cyclopteri has caused several instances of disease in both wild and farmed lumpfish (Mullins et al., 1994; Freeman et al., 2013; Alarcon et al., 2016). The parasite has been observed in the nuclei of lymphocytes or lymphoblasts (Mullins et al., 1994; Freeman et al., 2013), and may stimulate an unusual proliferation of these leukocytes (Karlsbakk et al., 2014). Lumpfish heavily infected with *N. cyclopteri* show characteristically enlarged kidneys with pale patches or nodules (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013; Karlsbakk et al., 2014; Alarcon et al., 2016). The parasite has previously been detected in kidney, heart, spleen, skin, gills and eggs of lumpfish using nested PCR (Freeman et al., 2013; Alarcon et al., 2016).

In the present study, the parasite density in different tissues were quantified in order to examine the tissue distribution of the parasite. *Nucleospora cyclopteri* was detected in all nine tissues examined: anterior-, mid- and posterior kidney, spleen, heart, gills, brain, muscle, liver and blood. This supports that the infection may be systemic, as previously suggested. Densities of *N. cyclopteri* were highest in anterior kidney, followed by mid and posterior kidney, spleen, heart and gills. The highest variation of parasite densities was detected in the anterior kidney, and this might be related to more extensive parasite proliferation at this site. Urine were positive for *N. cyclopteri* and spores were detected in kidney smears, indicating possible shedding of the parasite from the host kidney via urine.

It is also demonstrated that the parasite can be detected using skin, gill and vent swabs, blood samples and gill biopsies, and thus showing the possibility of non-lethal detection in lumpfish. The most promising non-lethal samples for detection were gill biopsies and leukocyte fractions from blood samples. Further work should be conducted in order to improve these detection methods further.

1. Introduction

1.1 The challenge with salmon louse

The impact of salmon louse (*Lepeophtheirus salmonis*) on wild salmonids is currently one of the most important issues facing the management of Norwegian aquaculture (Vollset et al., 2017). Salmon louse is an ectoparasite that feeds on mucus, skin tissue and blood of salmonids at sea, causing skin lesions that may lead to osmotic problems and secondary infections (Pike & Wadsworth, 1999; Finstad et al., 2000; Nolan et al., 2000; Bjørn et al., 2001; Heuch et al., 2005). The concentration of hosts within a salmon farming area is an important source of louse infestations on wild fish (Bjørn et al., 2011; Serra-Llinares et al., 2014; Serra-Llinares et al., 2016). In order to minimize the impact on wild fish, the aquaculture production capacity has to be regulated in accordance with the impact of louse on wild fish in a particular area (Produksjonsområdeforskriften, 2017, § 8). The legal limits for the maximum mean number of louse per fish in an aquaculture facility is 0.5 adult females, except during the smolt migration in spring when the limit is 0.2 (Forskrift om lakselusbekjempelse, 2012, § 8). Different control measures are in use to ensure that the farms do not exceed the legal limits, and the annual cost related to salmon louse control in Norway was recently estimated to be around 3-4 billion NOK (Iversen et al., 2015), not including loss of productivity. Traditionally, chemotherapeutants have been used for louse treatments, but reduced sensitivity to several of the chemicals used has been observed (Denholm et al., 2002; Grøntvedt et al., 2014). Therefore, treatments with drugs has decreased, while the use of mechanical treatments has increased (Litleskare, 2018). Mechanical treatment methods against louse vary from heated seawater, hosing, hosing combined with brushes and freshwater treatment (Holan et al., 2017). However, there are fish health and welfare challenges related to the use of mechanical treatments (Holan et al., 2017; Gismervik, 2018). Another form for louse treatment is the use of cleaner fish, which may be less stressful for farmed fish (Holan et al., 2017).

1.2 Cleaner fish

Different wrasse (Labridae) species and lumpfish *(Cyclopterus lumpus)* are shown to feed on salmon louse (Bjordal, 1988, 1990; Willumsen, 2001) and are therefore used as cleaner fish in fish farms (Mortensen, 2017). The number of cleaner fish used in Norwegian aquaculture has increased exponentially since 2008, and numbered around 36 million fish in 2016 (Fisheries,

2017). Traditionally wild-caught wrasse have been used (Mortensen et al., 2015), but the production of farmed lumpfish has increased massively in the last years, from 1.9 million produced in 2013 to 15 million in 2016 (Fisheries, 2017). Only a small portion of wrasse are farmed today; only about 0.4 million ballan wrasse were produced in 2016 (Fisheries, 2017). Farming of wrasse is more challenging in contrast to that of lumpfish, due to a long production cycle and the need for live feed (Mortensen et al., 2015; Øie et al., 2017; Powell et al., 2017). Another drawback with wrasse is that they do not feed at low temperatures (Sayer & Reader, 1996), which limits their use as cleaner fish over winter and in the northern parts of Norway. The use of cleaner fish can be seen as a more environmentally friendly method of delousing than chemical treatments. In contrast to mechanical treatments, it is not associated with stressful handling of the farmed salmonids (Holan et al., 2017). However, there are ethical and welfare issues related to the use of cleaner fish today, such as the significant loss of fish in sea cages due to escapes, predation and mortality (Mortensen, 2017; Gulla & Bornø, 2018). The use of wild-caught fish in particular also raises a number of ethical questions regarding wild fish in captivity and overfishing of the wild population (Holan et al., 2017).

1.3 Lumpfish

Lumpfish (*Cyclopterus lumpus* L.) is a member of the family lumpsuckers (Cyclopteridae). Traditionally lumpfish have been fished as a source of roe, processed and sold as caviar (Davenport, 1985). In 2000, wild-caught juvenile lumpfish were tested as cleaner fish on Atlantic Salmon (*Salmo salar*) at a research facility in Norway, with promising results (Willumsen, 2001). Imsland et al. (2014) demonstrated the grazing efficiency of lumpfish on salmon louse in salmon pens. The lumpfish significantly reduced the mean number of salmon louse attached to the salmon. Based on these findings, interest in the use of lumpfish for delousing has rocketed (Powell et al., 2017). Today there are 23 companies producing more than 16 million lumpfish in Norway (Directorate of Fisheries, 2017; Fisheries, 2017). Wild-caught lumpfish are used as brood fish, and the farming is considered relatively easy due to there being no need for live feed. After five to seven months, the juveniles are ready to be transferred to sea pens, where they are considered active throughout the year, only reducing grazing efficiency during high temperatures (Mortensen, 2017).

The mortality of farmed lumpfish is generally high, both during the production and in sea pens (Gulla & Bornø, 2018). The most important cause of mortality during the production and in sea pens is bacterial infections with the most severe being atypical furunculosis (atypical

Aeromonas salmonicida), pasteurellosis (*Pasteurella* sp.) and classical vibriosis (*Vibrio anguillarum* and *Vibrio ordalii*) (Bornø et al., 2016; Gulla & Bornø, 2018). External ulcerative lesions caused by *Moritella viscosa* and *Tenacibaculum* spp. are currently also a widespread problem (Småge et al., 2016; Gulla & Bornø, 2018). Piscirickettsiosis due to *Piscirickettsia salmonis* has newly been diagnosed in farmed juvenile lumpfish in Ireland (Marcos-López et al., 2017), but has not been reported for this host in Norway (Gulla & Bornø, 2018). Viral diseases have previously not been considered to be an important reason for the mortality in lumpfish (Bornø et al., 2016). However, a newly described virus named *Cyclopterus lumpus* virus (previously called Lumpfish flavivirus) is associated with liver pathology and moderate to high mortality both in sea pens and during the production of lumpfish (Vestvik et al., 2017; Gulla & Bornø, 2018; Skoge et al., 2018). Viral haemorrhagic septicaemia virus (VHSV) infections (genotype IVd) were detected in wild-caught lumpfish brood stock in Iceland (Jónsson, 2016; Cuenca, 2017), but have not been reported for this host in Norway (Gulla & Bornø, 2018). Viral haemorrhagic septicaemia (VHS) is a notifiable disease and detection of the causative virus would result in loss of disease-free status in Norway (Omsetnings- og sykdomsforskriften for akvatiske dyr, 2008, vedlegg 1 & 2). A new ranavirus has been isolated from reared juvenile and broodfish lumpfish at multiple locations in the north Atlantic area, but it is not known if the virus causes disease (ICES, 2017; Stagg et al., 2017).

Several species of eukaryotic parasites are known from lumpfish, mostly from wild fish, and the importance of many of these species is still uncertain (Karlsbakk et al., 2014; Gulla & Bornø, 2018). *Gyrodactylus* sp. have been detected in farmed juvenile lumpfish with skin lesions (Mortensen, 2017) and in gills of lumpfish in sea pens showing chronic gill inflammation (Alarcon et al., 2016), however the importance of the parasite in lumpfish is not known (Gulla & Bornø, 2018). Lumpfish in sea pens can have heavy infestations of the ectoparasite *Caligus elongatus*, and delousing of lumpfish against this parasite has been reported (Mortensen, 2017). Amoebic gill disease (AGD) due to *Paramoeba perurans* has also been reported as an important challenge during the production of lumpfish and in sea pens (Gulla & Bornø, 2018). The parasite is especially important in regards to the risk of transmission between lumpfish and salmon in sea pens (Mortensen, 2017). A challenge experiment has shown that lumpfish can transfer amoebae to salmon, and lumpfish seem less susceptible and develop disease more slowly compared to salmon (Haugland et al., 2017). The microsporidian parasite *Tetramicra brevifilum* has caused disease in wild-caught broodfish held at a facility in Ireland, presenting with whitish nodules (xenomas) in most organs and tissues, inflammation, degeneration and necrosis (Scholz et al., 2017). Another microsporidian,

Nucleospora cyclopteri has caused several instances of disease in both wild and farmed lumpfish (Mullins et al., 1994; Freeman et al., 2013; Alarcon et al., 2016).

1.4 Microsporidia

Microsporidians are obligate intracellular parasites infecting a variety of animal hosts (Lom & Dyková, 1992; Lom, 2002). They lack mitochondria and can only develop and proliferate inside other cells (Vávra & Larsson, 1991). Microsporidians produce small, thick-walled, environmentally resistant spores (usually under 7 μm in diameter), that can retain their viability in water for at least one year (Lom & Dyková, 1992). The spore consists of sporoplasm, the infectious germ, and a sophisticated extrusion apparatus, consisting of a polar tube (Vávra & Larsson, 1991). Under appropriate conditions inside a suitable host, the polar tube can inject the sporoplasm into a host cell. Inside the cell the germ starts a proliferative cycle, called merogony, and eventually the development of spores, called sporogony. Microsporidians typically develop within the cytoplasm of the host cell. An exception to this is seen in members of the genus *Nucleospora* (syn. *Enterocytozoon*) (Lom & Nilsen, 2003).

1.5 *Nucleospora* spp.

Nucleospora spp. possess many unique features not present in any other microsporidia infecting fish. One of them is the intranuclear development (Lom & Nilsen, 2003). All *Nucleospora* species described so far infect fish, including *N. salmonis* in various salmonid species (see below), *N. cyclopteri* in lumpfish (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013) and *N.* sp. in Atlantic halibut (*Hippoglossus hippoglossus*) (Nilsen et al., 1995). *Nucleospora salmonis* was first identified in Chinook salmon (*Oncorhynchus tshawytscha*) in North America and was associated with acute anemia, leukemia and lymphoblastosis (Elston et al., 1987; Hedrick et al., 1990; Morrison et al., 1990). The parasite infects the nuclei of lymphoblasts (Chilmonczyk et al., 1991) causing an abnormal proliferation of hematopoietic tissue (Hedrick et al., 1990; Baxa-Antonio et al., 1992). Signs of disease are marked gill pallor, exophthalmia, enlarged spleen, kidney hypertrophy, swelling of the posterior intestine and ascites (Hedrick et al., 1990). Since the first reports in North America, *N. salmonis* has also been detected in France (Chilmonczyk et al., 1991) and Chile (Kent et al., 1995; Gresoviac et al., 2000), and in various salmonid species: coho salmon (*Oncorhynchus kisutch*) (Kent et al., 1995), rainbow trout (*Oncorhynchus mykiss*) (Chilmonczyk et al., 1991; Georgiadis et al., 1998), Atlantic salmon (*Salmo salar*) (Kent et al.,

1996), brook trout (*Salvelinus fontinalis*) and lake trout (*Salvelinus namaycush*) (Gresoviac et al., 2000). The complete life cycle of *N. salmonis* is not known, but likely involves transmission routes and persistence in host populations by horizontal, vertical or both types of parasite transmission (Hedrick et al., 2012). Horizontal transmission of *N. salmonis* has been experimentally demonstrated by intraperitoneal injections of infected cells (Hedrick et al., 1990), by feeding infected tissues to uninfected fish and by cohabitation of uninfected fish with experimentally infected fish (Baxa-Antonio et al., 1992). Oral ingestion of spores is presumed to be the route of entry of the infective stage (Barlough et al., 1995). The parasite then spreads from this initial site to the major cellular targets in hematopoietic tissues, principally kidney and spleen (Morrison et al., 1990; Hedrick et al., 1991). *Nucleospora salmonis* has also been detected in faeces of infected fish by PCR (Barlough et al., 1995) and in hindgut using qPCR (Foltz et al., 2009), suggesting a role of the intestine in shedding of the parasite. Infections among spawning Chinook salmon and the appearance of *N. salmonis* infections in their progeny provided evidence suggesting vertical transmission (Hedrick et al., 2012). This is further supported by the finding of *N. salmonis* in Chile (Kent et al., 1995; Gresoviac et al., 2000) since salmonids are not native to the southern hemisphere (Gajardo & Laikre, 2003) and salmonid eggs have been repeatedly imported to Chile from areas where *N. salmonis* occurs (Bjørndal & Aarland, 1999). During experimental infection studies, *N. salmonis* has been detected in several tissues indicating a systemic spread of infected lymphocytes in the host (Hedrick et al., 1991; Barlough et al., 1995). In vitro studies by Wongtavatchai et al. (1995) provided evidence for immune depression, as both the B- and T-cell functions of isolated lymphocytes from *N. salmonis*-infected Chinook salmon were reduced. The humoral response was suppressed, and the response to several mitogens including lipopolysaccharide (LPS) was impaired in infected fish. Secondary infections with bacteria are common among fish with *N. salmonis* infections, and immunosuppression due to this microsporidian could be responsible for increased susceptibility of the host to other pathogens.

1.6 *Nucleospora cyclopteri*

The first observation of *Nucleospora* sp. infection in lumpfish was by Mullins et al. (1994). They reported an intranuclear *Enterocytozoon* sp. (a synonym of *Nucleospora*) in the nuclei of lymphoblasts and lymphocytes of cultured juvenile lumpfish experiencing chronic mortalities. Later Freeman et al. (2013) described *Nucleospora cyclopteri* from wild Icelandic lumpfish with various degrees of macroscopic pathology, most prominent in the kidneys which usually

were swollen (renomegaly). Freeman and Kristmundsson (2013) studied the ultrastructure of the microsporidian spores and concluded that the parasites observed by Mullins et al. (1994) were *N. cyclopteri*. Affected cells are lymphocytes and lymphoblasts, and the parasite is situated in the nuclei of these cells (Mullins et al., 1994; Freeman et al., 2013). Before the formation of spores, the parasite may stimulate an unusual proliferation of leukocytes (Fig. 1A), causing renomegaly (Fig. 1B) (Karlsbakk et al., 2014). Lumpfish infected with *N. cyclopteri* show characteristically enlarged kidneys with pale patches or nodules, signs of anaemia and often exophthalmia (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013; Karlsbakk et al., 2014; Alarcon et al., 2016).

The spores produced inside the leukocyte nuclei are minute; in transmission electron microscope (TEM) images 2.1 to 2.5 µm in length and 1.0 µm in width (Mullins et al., 1994; Freeman & Kristmundsson, 2013), or about 3.1 x 1.3 µm when measured in fresh kidney smears (Freeman et al., 2013).

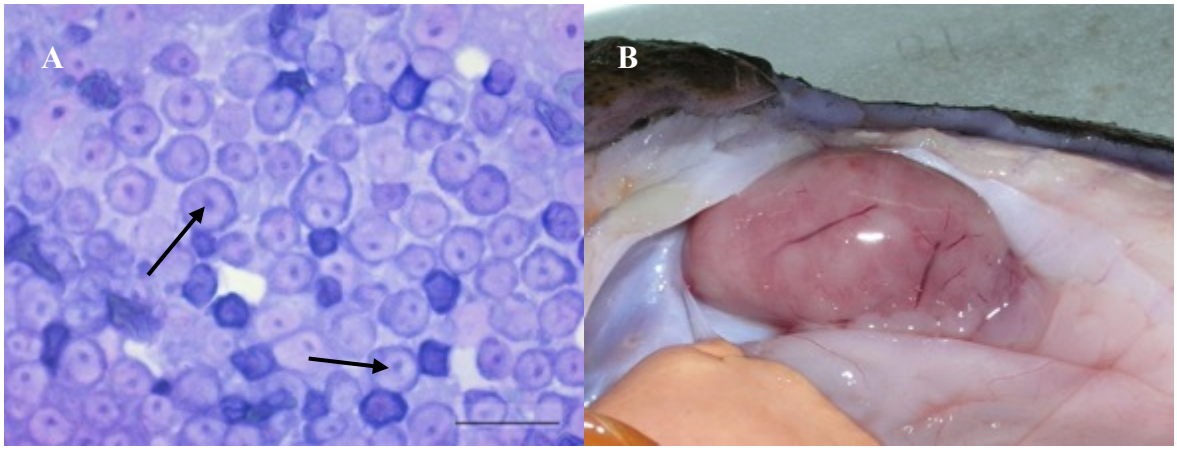

Figure 1. A: Proliferation of infected lymphocytes in Diff-Quick stained kidney imprint from a wild lumpfish, Norway. Pre-spore stages (arrows) inside the cell nuclei. Scalebar is 20 µm. **B**: Extensive renomegaly and pallor of anterior kidney, from the same fish as A. Photo 1B from Karlsbakk et al. (2014).

Nucleospora cyclopteri has been found on numerous occasions in wild lumpfish in Norway (Karlsbakk et al., 2014), and is thought to be the most widespread pathogen in wild-caught lumpfish (Lein et al., 2017). The first report of the parasite in farmed lumpfish in Norway was in 2013 in a case with increased mortality of lumpfish in sea pens with salmon (Alarcon et al., 2016). *Nucleospora cyclopteri* was detected in gills and heart in fish using PCR, and spores were revealed in kidney, spleen, and to a lesser extent in gills, heart, liver and intestine (Alarcon et al., 2016). The parasite has also been detected in skin and eggs, the latter suggesting vertical

transmission (Freeman et al., 2013). In order to prevent the possible transmission of the parasite to offspring, wild lumpfish used as broodfish are often screened for the parasite using qPCR (Glosvik, 2017; Kui, 2017). Kidney, milt or ovarian fluid are then analysed, and the parasite has been detected in all these sample types, as well as the hatchery-reared offspring (Kui, 2017). In disinfection trials with lumpfish eggs, it has been found that *N. cyclopteri* is difficult to kill using standard disinfection (Lein et al., 2017). There is currently no licensed therapeutic treatment for microsporidians in fish (Derome et al., 2016). However, an orally administered Fumagillin analogue (TNP-70) has shown some efficacy at controlling mortalities in Chinook salmon infected with *N. salmonis* (Higgins et al., 1998). Development of commercial anti-parasite vaccines has proven to be difficult, due to the complex structure and life cycle of parasites (Magnadottir, 2010). Immunization of naive rainbow trout with dead spores of *Loma salmonae* resulted in a protective cell-mediated immune response when re-challenged with live spores (Rodriguez-Tovar et al., 2006), thus demonstrating the possibility of vaccinating against microsporidians. Nevertheless, until an effective disinfection, treatment or vaccine is available, exclusion of carriers of *N. cyclopteri* offers the best possible prophylaxis.

There is a possibility that *N. cyclopteri* has an immunosuppressive effect on lumpfish, as the parasite appears to infect the same immune cells as *N. salmonis* and causes a similar disease. If so, then this microsporidian may be one of the factors contributing to the high mortality in farmed lumpfish in sea pens with salmonids, directly or by rendering the host more susceptible to other infectious agents such as bacteria and/or virus.

1.6 Non-lethal screening

Today, the intensive production of lumpfish relies on wild-caught brood fish stripped to obtain gametes (Vargas, 2015; Powell et al., 2017). To ensure a stable and sustainable source of lumpfish in the future, it will be advantageous to rear brood stock in captivity (Powell et al., 2017). Imsland et al. (2016) found significant differences in salmon louse grazing activity and disease resistance between distinctly different genetic families of lumpfish, thus suggesting possible traits that can be selected for in future breeding programs for lumpfish. The mentioned traits are the main breeding goals for lumpfish (Mommens, 2017), and breeding programs are currently in progress in Norway (Iversen, 2018). It will be important to detect carriers of *N. cyclopteri* in future breeding programs using non-lethal sampling methods, particularly if these are to be based on survivors from pens at the end of a production cycle, e.g. individuals with pronounced cleaning behaviour. Infections with *N. cyclopteri* may cause problems since the

parasite may be vertically transmitted, may have immunosuppressive effects on the host and also cause disease. Therefore, one of the aims of the present work was to identify non-lethal sampling methods to detect *N. cyclopteri* infections in lumpfish.

A previous study has shown that gill biopsy is a suitable non-lethal sampling method for the detection of *N. salmonis* in salmonids (Badil et al., 2011). Kidney biopsy can be used to detect infections with the bacterium *Francisella noatunensis*, the agent of francisellosis, in live Atlantic cod (*Gadus morhua*) used as broodfish (Isaksen et al., 2009). Blood sampling has been used to detect several pathogens in fish (Altinok et al., 2001; Lopez-Vazquez et al., 2006; Lopez-Jimena et al., 2010). Swabbing different areas of the fish has also been demonstrated as a non-invasive sampling method to detect several pathogens in Atlantic salmon. Infectious salmon anaemia virus (ISAV) has been detected by the swabbing of gill mucus (Griffiths & Melville, 2000) and by swabbing gills and fins (Aamelfot et al., 2016). However, non-lethal screening of IPNV using blood sampling and swabbing of gill, skin and rectum was not successful (Munro & Ellis, 2008).

In the present study, non-lethal samples were investigated: swabs, blood samples (including leukocyte fractions and leukocrit) and gill biopsies. When examining lumpfish for *N. cyclopteri* infections using non-lethal sampling, the results need to be related to a 'true' prevalence of the parasite. Currently, little is known about the importance of different tissues as sites for *N. cyclopteri* infection, with no quantitative analyses. Therefore, one of the aims was to study the tissue tropism of *N. cyclopteri*.

In 2017, the Norwegian Seafood Research Fund (FHF) initiated the project '*Nucleospora cyclopteri* in lumpfish; Occurrence, transmission, clinical importance and impact on cleaning behaviour' (project no. 901320). This project aims at increasing the current knowledge of the microsporidian parasite *Nucleospora cyclopteri* in lumpfish, including tissue tropism and non-lethal detection.

1.7 Aims of the study

The production of lumpfish needs to rely on a closed breeding cycle in captivity to ensure a stable and sustainable source of juveniles for stocking with salmon in the future. When breeding lumpfish, infections with *N. cyclopteri* may cause problems since the parasite may be vertically transmitted, may have immunosuppressive effects on the host and also cause disease. Therefore, *N. cyclopteri* infections in brood fish candidates need to be detected using non-lethal sampling methods. However, the development of such methods requires knowledge about

tissue distribution and the ‘true’ prevalence of the parasite. Therefore, there are two main aims for the study:

1. Study the tissue tropism of *N. cyclopteri* in wild-caught lumpfish, using quantitative real-time PCR.

2. Identify suitable and effective non-lethal sampling methods for the detection of *N. cyclopteri* infections in lumpfish.

2. Materials and Methods

2.1 Fish

A total of 41 wild-caught mature lumpfish were examined in the present study (Table 1). Sampling was carried out in June 2017 (n = 11) and November 2017 (n = 30). The June sample was taken during stripping, but this strategy proved to be too slow to obtain a sufficient number of samples. Therefore, we obtained a separate group of fish for the study when sampling in November. All fish were caught in shallow water in the area Sveggevika-Ramnfjorden, Averøy, Møre and Romsdal, Norway, using nets with a mesh size of 267 mm. The fish were landed at Skjerneset, Ekkilsøy and kept in a tank (500 L) on land, with a constant flow of sea water until sampling.

Table 1. Details of the wild lumpfish landed at Ekkilsøy, Møre and Romsdal county. n: number of individuals sampled. Mean length (cm) and weight (g) are given for each sampling, and the ranges (min. – max.) are given in brackets.

Sampling	n	Sex	Length (Range)	Weight (Range)
June 2017	11	Female	44.5 (41.0–48.0)	2684 (1600–3603)
November 2017	11	Male	35.1 (30.6–41.8)	1480 (875–2101)
	19	Female	45.0 (40.5–49.5)	3194 (2209–5196)

2.2 Sampling

Each fish was killed by a blow to the head and placed on a sterile sheet on its right side facing left before the swab sampling was performed. Standard cotton swabs (Soft Style Bomullspinner, Lemoine) were rubbed (while rotated) against different areas of the skin, the gill and the vent of the fish for approximately 10 seconds. Six different sites were swabbed and collected in the following order (Fig. 2):

1. An area of about 1x5 cm on the skin at the dorsal side of the fish.
2. The area behind the pectoral fin (‘BP’), covering both fin base and adjacent skin.
3. The opercular margin (‘OM’) was swabbed by moving the swab just underneath the opercular margin and pressing the opercular down, covering both the inner surface of the opercular margin and the opposing part of the body.

4. The sucker was swabbed in an area of 1x5 cm along the medial diameter of the sucking disk.
5. The gills were swabbed by moving the swab over the filaments of the hemibranchs of the first and second gill arch on the left side.
6. The vent was swabbed by inserting the cotton end and rotating it.

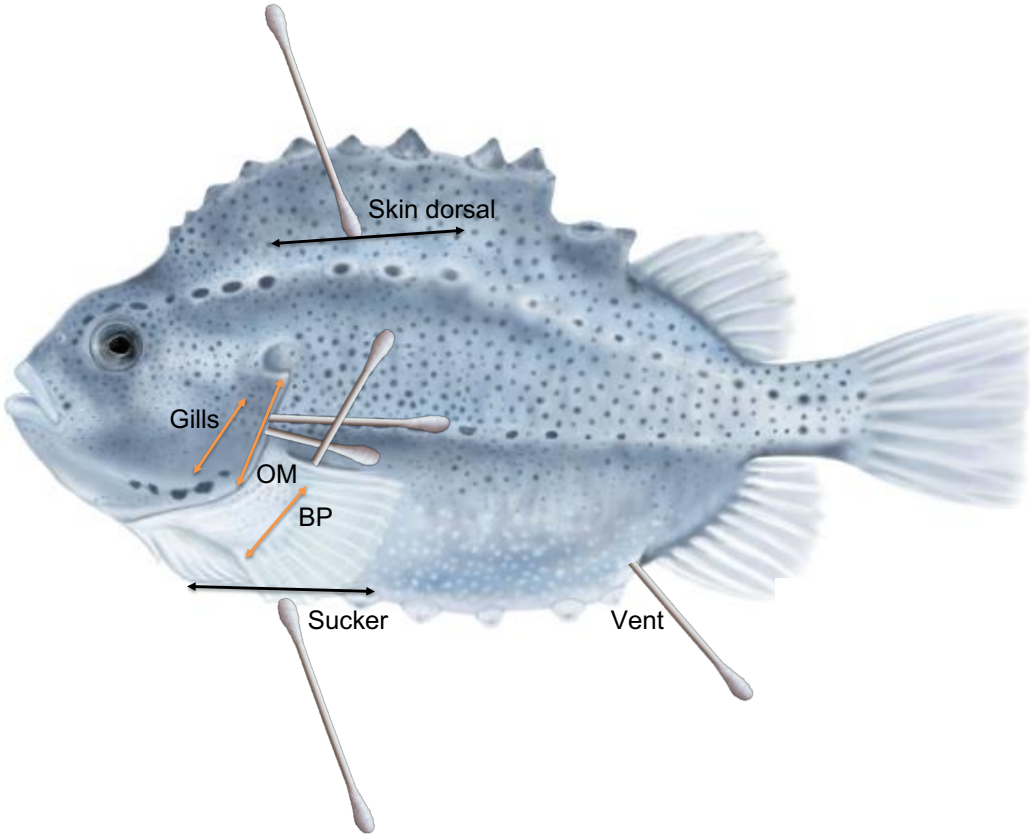

Figure 2. The six swabbing sites on the lumpfish. Black arrows indicate sites on skin. Orange arrows indicate areas swabbed underneath opercular and pectoral fin. Abbreviations: BP behind pectoral fin, OM opercular margin.

To account for a possible *N. cyclopteri* signal from the fish water, some control swabs were included that were simply dipped in the tank water. In addition, swabs were taken from the biofilm on the tank walls near the water surface. These water and tank samples were taken after both sampling days in November. All samples were stored in individual cryotubes with 0.5 mL RNA preservation solution (RNAps). These were kept at room temperature for 24 hours, followed by storage at -20 °C prior to RNA extractions.

Immediately after swabbing the fish, wet weight was recorded in grams (g), and total fish length was measured in centimetres (cm). Any gross signs observed were recorded. Blood samples were collected from the caudal vessels using a sterile needle (Venoject, 0.7 x 25 mm) and 3

mL EDTA-containing evacuated blood collection tubes (Venoject, 10 x 65 mm). These were stored at 4 °C before further sample processing the same day (See section 2.3). A necropsy was then performed using sterile techniques. Sex was determined, and any internal gross pathology was recorded. A 3 ml urine sample was collected from the urinary bladder using a sterile needle (Venoject, 0.7 x 25 mm) and 3 mL evacuated blood collection tube (Venoject, 10 x 65 mm) (no additive), and stored overnight at 4 °C to allow sedimentation. Five drops (approx. 250 µL) were then pipetted from the bottom (including any sediments) of the urine sample tubes and were stored in RNAps as described previously. Fish with no urine in their bladder were not sampled (n = 4). Ovarian fluid and milt samples were also taken. These were analysed by PatoGen Analyse AS using the same assays (qPCR, see below), as a separate part of the parent FHF project. Tissues were sampled aseptically from the following organs; anterior-, mid- and posterior kidney (Fig. 3), spleen, liver, heart, brain (medulla oblongata), muscle and gills. All tissues were collected in triplicate and of standardized size (2 x 2 mm). During sampling of kidneys, imprints were made from the anterior-, mid- and posterior kidney for later staining (See section 2.4) and visualization of infected cells and spore detection. Bile was collected from the gall bladder using 1 ml sterile syringes (disposable, no additives) and 5 drops were stored as previously described. Fish with no bile in their gall bladder were not sampled (n = 13). The anterior kidneys of all fish were photographed to document any appearance of potential correlation with *N. cyclopteri* infections.

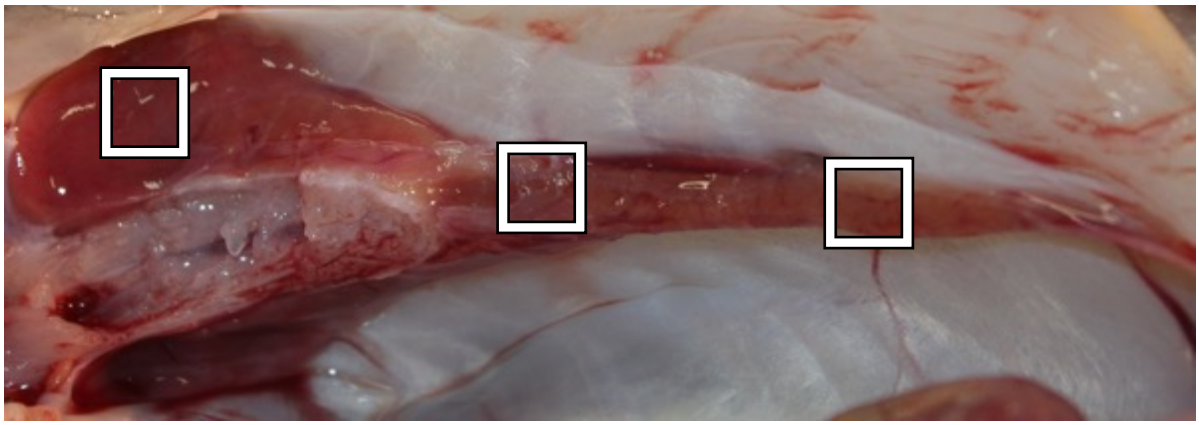

Figure 3. The location of kidney samples (squares) from left to right: anterior, mid and posterior kidney.

2.3 Blood samples

Hematocrit was determined by transferring blood to heparinized microhematocrit tubes and centrifuging (16000 x g, 3 min at room temperature) using a micro-hematocrit centrifuge (Hettich, Germany). The hematocrit (erythrocrit and leukocrit) was then measured. Five drops

of blood (approx. 250 µL) were stored as previously described. Leukocytes were separated from blood by transferring 1 mL blood to a 1.5 mL microtube and centrifufing at 2000 x g for 1 min at room temperature. The visible buffy coat/leukocyte fraction was then removed and five drops (approx. 250 µL) were stored. Thin blood and leukocyte smears were made from the June samples (n = 11).

2.4 Staining procedure for kidney imprints, blood and leukocyte smears

Hemacolor Rapid staining (Sigma-Aldrich, Germany) was used to stain all the slides (kidney imprints, blood and leukocyte smears) according to the manufacturer´s instructions, but using half the immersion time for the blue colour reagent. Preparations were first fixed by dipping the slide 5 x 1 sec in the fixing solution. Next, the slides were dipped 3 x 1 sec in the red colour reagent, and finally 3 x 1 sec in the blue colour reagent. The slides were rinsed 2 x 10 sec in a buffer solution pH 7.2, air-dried, and cover slipped. The presence of infected cells and spores was evaluated with an Axio Scope A1 light microscope (Zeiss, Germany), using bright field microscopy at 400 x and 1000 x (oil-immersion) magnification. Digital images were taken using Axiocam 105 colour camera (Zeiss, Germany). Measurements of spores were taken from the images using Image J (v 1.52, National Institute of Health, USA).

2.5 RNA extractions

2.5.1 Tissues

Total RNA from collected tissues were extracted using TRI Reagent (Sigma-Aldrich, Germany) as per manufacturer's instructions, with some modifications. Tissue samples were transferred to 2.0 mL safe-lock microtubes (Sigma-Aldrich, Germany) containing 1 ml TRI Reagent and 5 mm stainless steel beads, and were homogenized using the TissueLyser II (Qiagen, Germany) for 3 minutes at 30 Hz. The samples were spun down using a micro-centrifuge (VWR, Norway) and allowed to stand for 5 minutes at room temperature. For the phase separation, 200 µL chloroform (Sigma-Aldrich, Germany) was added to the homogenized sample before being shaken vigorously for 15 seconds and allowed to stand for 2 minutes at room temperature before centrifugation (Thermo Fisher Scientific, USA) (21100 x g, 15 min at 4 °C). The mixture separated into a red organic phase, an interphase and a colourless upper aqueous phase containing RNA. To isolate the RNA, 450 µL of the aqueous phase was transferred to 500 µL of 2-propanol (Isopropanol, Kemetyl, Norway) in a clean 1.5

mL microtube (Sigma-Aldrich, Germany), mixed and allowed to stand for 5 minutes at room temperature, before centrifugation (21100 x g, 10 min at 4 °C) to pellet the RNA on the bottom and side of the tube. The RNA was washed twice with 1 mL and 0.5 mL 75% ethanol (Honeywell, Germany) during centrifugation (21100 x g, 5 min at 4 °C). After drying, the RNA pellet was dissolved in 20-100 µL RNase-free water (Milli-Q, USA) heated to 60 °C. The purity and concentration of RNA was tested with NanoDrop ND-100 spectrophometer (Thermo Fisher Scientific, USA), and samples with a concentration over 1000 ng/µL were diluted to approximately 1000 ng/µL. The samples were stored at -20 °C prior to quantitative real-time PCR analysis.

2.5.2 Blood, leukocytes, bile and urine

Prior to RNA extraction from blood, leukocytes, bile and urine, the sample suspended in RNAps was transferred to individual 2.0 mL safe-lock microtubes. After centrifugation (21100 x g, 10 min at 4 °C) to pellet the material, the RNAps was transferred back to the original cryotube stored at -20 °C as a back-up. The sample was homogenized in 0.5 mL TRI Reagent by vortexing (Biosan, Latvia). For the phase separation and RNA isolation, half of the amount of the respective reagents were used, using 0.5 mL of TRI Reagent. The following procedures were performed as previously described for the tissues.

2.5.3 Swabs

A pilot experiment was conducted to find an effective method to release the material from the swabs into the RNAps. Lumpfish independent from this study were swabbed, and three different methods were used to release material from swabs. The isolated RNA was screened using quantitative real-time PCR, and the results were inconsistent. Therefore, the easiest and least time-consuming method was chosen.

Prior to RNA extraction from swabs, the samples were vortexed a few seconds to release material from the cotton swab into the RNAps (Fig. 4). The RNAps was transferred to a 2.0 mL safe-lock microtube and centrifuged (21100 x g, 5 min at 15 °C) to pellet material. About 20 µL of the sample, including any pelleted material, was left in the microtube and the rest was transferred back to the original cryotube with the swab and stored at -20 °C as a back-up. RNA was then extracted using the same protocol as described for blood, leukocytes, bile and urine.

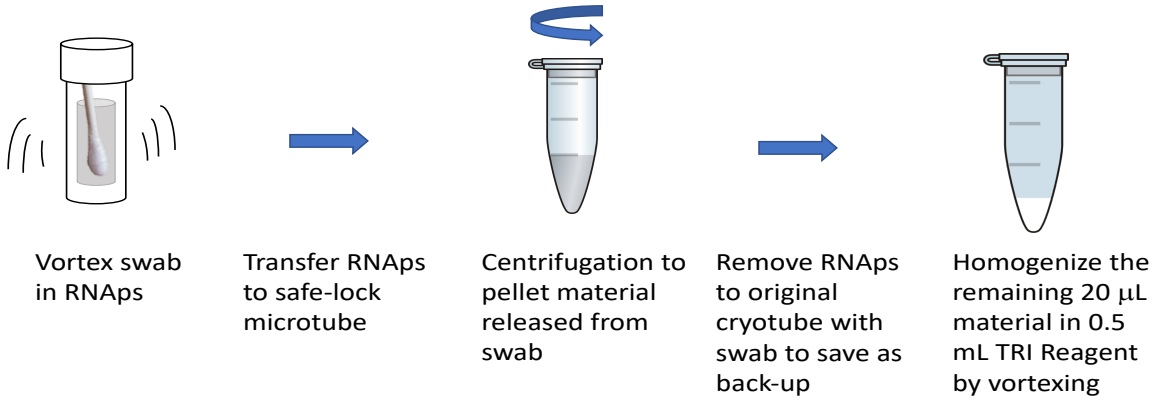

Figure 4. Procedures prior to RNA extraction from swabs.

2.8 Quantitative real-time PCR (qPCR)

All the different RNA samples processed were screened for *N. cyclopteri* RNA using quantitative real-time PCR (qPCR). A specific assay targeting the 16S ribosomal RNA gene for *N. cyclopteri* (Nuc) was used. Lumpfish elongation factor 1a (Ef 1a) assay was used as a reference gene to normalize the results for RNA input. Primer and probe sequences of the assays are listed in Table 2. The AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, USA) was used according to manufacturer's instructions in 96 well plates, but in a 10 µL volume (Table. 3). All analyses were run using either Applied Biosystems 7500 Real-Time PCR System or QuantStudio 3 (Applied Biosystems, USA) following standard AgPath conditions: 10 min of 45 ºC and 10 min of 95 ºC, followed by 45 cycles of 15 seconds of 95 ºC and 45 seconds of 60 ºC. The amplification curves were analysed by use of 7500 Software (v 2.0.6, Applied Biosystems, USA) or QuantStudio Software (v 1.4, Applied Biosystems, USA), and the threshold was set as 0.1 for both assays. RNA extraction control (REC), non-template control (NTC) and positive controls were included for both assays on every plate.

Table 2. Primer and probe sequences of assays used in the study. The sequences were kindly provided by PatoGen Analyse AS.

Target (Assay name)	Primer and probe sequences (5′-3′)	
Lumpfish Elongation factor 1a (Ef 1a)	Forward	AGTCCGTAGTCGTAGATGCAATTAAA
	Reverse	GCTCCGCCACAATTCAACA
	Probe	6FAM-TATGGATCAAGCATACTAAG
Nucleospora cyclopteri 16S (Nuc)	Forward	GTTGAGACCGGCATCATCAA
	Reverse	AGGTTGCAGGGAGCAAAGG
	Probe	6FAM-CCCACCATGGTCGTC

Table 3. Quantitative real-time PCR set up.

Reagents	Volume (µl)	Final concentrations
2X RT-PCR Buffer	5.00	-
Forward primer 10 uM	0.40	400 nM
Reverse primer 10 uM	0.40	400 nM
Probe	0.12	120 nM
25X RT-PCR enzyme mix	0.40	-
Nuclease-free water	1.68	-
Template	2.00	-
Total	10.00	-

2.9 Assay validation and relative quantification

The efficiencies of the assays used in the qPCR reactions were tested to determine the assays' ability to detect different amounts of the target gene. Standard curves based on tenfold dilutions of known templates were performed for both assays. The dilution series were run in triplicate with 6 replicates for the final dilution (Appendix I). Mean threshold cycle (Ct) of the final dilution was set as the detection limit of the assay. Standard curves were generated on Microsoft Excel® 2017 by plotting Ct-values against the $\log_{10}$ of the RNA concentration using linear regression analysis. The efficiencies (E) of the assays were calculated using the following formula:

$E = 10^{-1/slope}$ (Pfaffl et al., 2004).

The maximal efficiency of an assay is E = 2 where every single template is replicated in each cycle, and the minimal value is E = 1, corresponding to no replication (Pfaffl et al., 2004). In

order to quantify the amount of RNA of the target gene relative to target RNA in other samples and other tissues, normalized expression (NE) was calculated using the following formula:

$$NE = \frac{(E_{reference})^{CT\ reference}}{(E_{target})^{CT\ target}}$$ (Simon, 2003)

Normalized expression (NE) of *N. cycloperi* RNA was used as a measure of parasite density. Density is the number of individuals of an agent in a measured sampling unit taken from a host, e.g., in units of area, volume, or weight (Bush et al., 1997). The density (NE) in negative samples was set at '0'. In order to obtain a good measure for parasite density in individual fish, an 'Individual mean density' was calculated as the mean density of the kidney parts, spleen and heart (the principal tissues (see below)). A similar expression of the reference gene (Ef 1a) is necessary for the NE to be comparable across tissue types. This was not always the case in our study. Hence parasite density had to be approximated in a different way, so here we used a measure based on the Ct-values, called parasite 'load'. Load was calculated as the number of cycles in the qPCR run minus the Ct-value (45-Ct). This measure may vary with sample size, but since our aim was to study tissue tropism, equal sample size was always attempted. Also, mean values should be little affected by random variation in size. Analyses of density based on NE was also done based on load, in order to ascertain that any bias due to the reference gene did not affect the results.

Prevalence is the proportion of hosts in a sample or population infected with a particular parasite (Bush et al., 1997). In the present case, a lumpfish was considered infected if two or more of the samples from the principal tissues were within the detection limit (see section 3.1). These tissues were chosen based on the results of the tissue tropism study: anterior-, mid- or posterior kidney, heart and spleen (see section 3.4).

2.10 Statistical analyses

Prevalence in two samples was compared using Fisher's exact tests (FET). Parasite densities were not normally distributed, so two independent samples were compared using non-parametric Mann-Whitney U-tests (MW). For the same reason, a Kruskal-Wallis test (KW) was used to compare Ct-values of Ef 1a between tissues. A post-hoc multiple comparisons (MC) test was used to identify where the significant variation occurred. Tissues with deviating Ef 1a expression were not compared with other tissues based on NE. Analyses based on load were also calculated, as confirmatory tests for the analyses based on NE, but independent of Ef 1a expression. Tissues with low Ef 1a expression were included in the load analyses on the

assumption that the examined tissue samples were of comparable size. A Friedman's ANOVA by ranks comparing multiple dependent variables was used to examine parasite densities across tissues with comparable Ef 1a expression. Post-hoc testing was done with Signs tests, employing Bonferroni corrections for robustness. Correlations were examined using Spearman's rank correlation coefficients (r_s). Double zeroes were deleted in these analyses. All statistical tests were performed with Statistica 64 (v 13.3. Dell Inc, Tulsa, USA).

3. Results

3.1 Standard curves and amplification efficiencies of the qPCR assays

Efficiency tests were performed on the specific assay for the target gene, the 16S ribosomal RNA gene of *N. cyclopteri* (Nuc), and the assay for the reference gene, Elongation factor 1a (Ef 1a). The standard curve for Nuc (Fig. 5) had a regression line with a slope of -3.455, generating an amplification efficiency of E = 1.947. The detection limit for the Nuc assay was 35.4. The standard curve for Ef 1a (Fig. 6) had a regression line with a slope of -3.6340, corresponding to an amplification efficiency of E = 1.883.

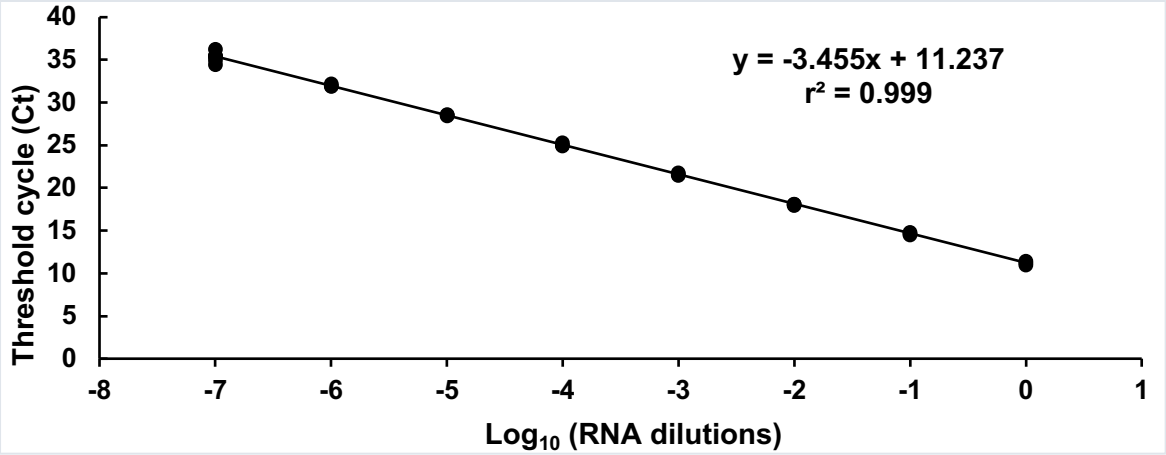

Figure 5. Standard curve for the target gene assay (Nuc), the 16S ribosomal RNA gene of Nucleospora cyclopteri. Amplification efficiency of E = 1.947.

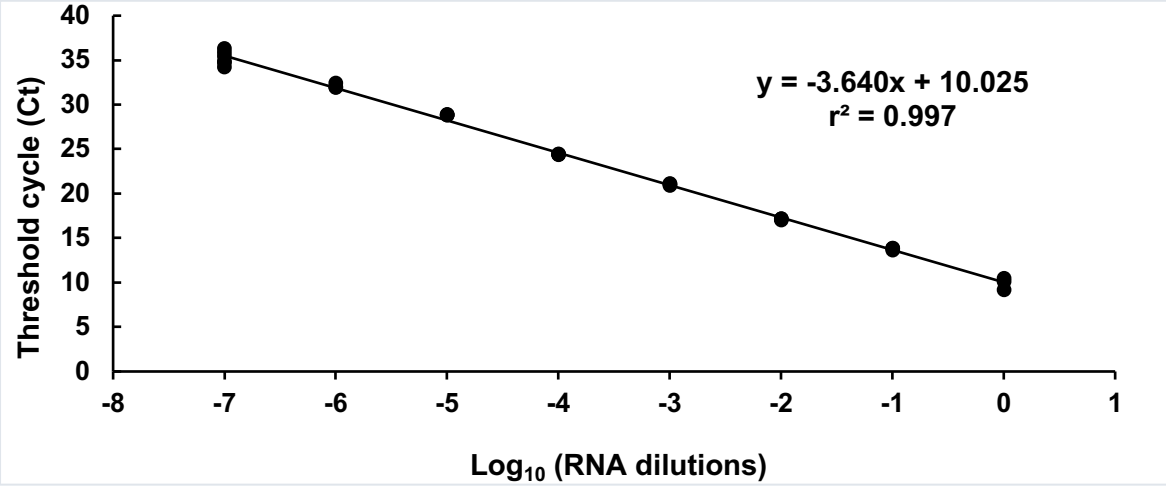

Figure 6. Standard curve for the reference gene, elongation factor 1a (Ef 1a) assay. Amplification efficiency of E = 1.883.

3.2 Macroscopic pathology

The most prominent macroscopic pathology was observed in the kidney, and especially in the anterior kidney. There was a variation on the extent of the pathology between individual fish. Mild renomegaly was observed in 5 fish and 3 fish had patchy pallor, thus together 20% of all the 41 fish had signs in the present study. Figure 7 (A-B) shows severe gross pathology for *N. cyclopteri*. The three fish from this study with the highest *N. cyclopteri* densities in anterior kidney, are shown in Figure 7 (D-F). These three fish also contained the spore stage of the parasites (see section 3.7). Normal kidney (uninfected) is shown for comparison (7C).

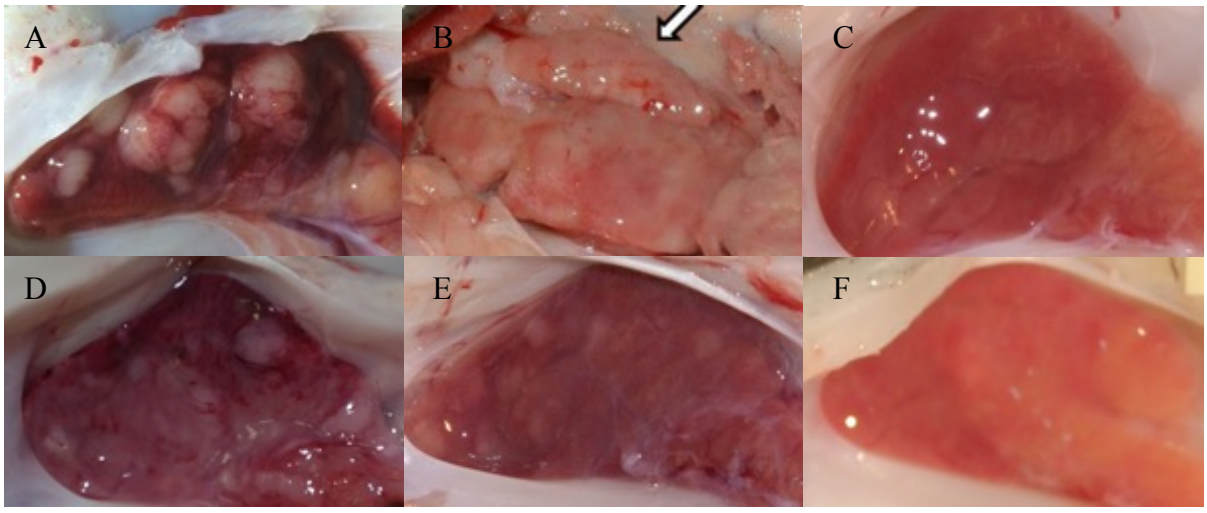

Figure 7. Anterior kidney appearance of wild-caught lumpfish, with various degrees of *Nucleospora cyclopteri* infection (**A, B, D, E, F**) and negative for this parasite (**C**). **A**: Large pale nodules (Ct: 7.2). Photo taken by Leon Stranden, Marine Harvest Vanylven. **B**: Extensive enlargement and pallor of the anterior kidney. Photo from Freeman et al. (2013). **C**: Normal anterior kidney, smooth and evenly red (uninfected). **D**: Patchy pallor (Ct: 10.9). **E**: patchy pallor (Ct: 7.6). **F**: Pale appearance (Ct: 11.9).

3.3 Prevalence of infection

A total of 41 lumpfish were screened for *Nucleospora cyclopteri* RNA in different tissue samples. Prevalence of infection was estimated based on two tissues among kidneys, spleen and heart positive. The prevalence was 59% for all fish, and 55% and 36% for females and males respectively (FET, $P = 0.15$) (Fig. 8). The June sample had a significantly higher prevalence compared to November (FET, $P < 0.001$).

Lumpfish egg-producers/hatcheries commonly analyse anterior kidney samples in order to reveal *N. cyclopteri* infections. If this was applied, the prevalence would be 76% for all fish.

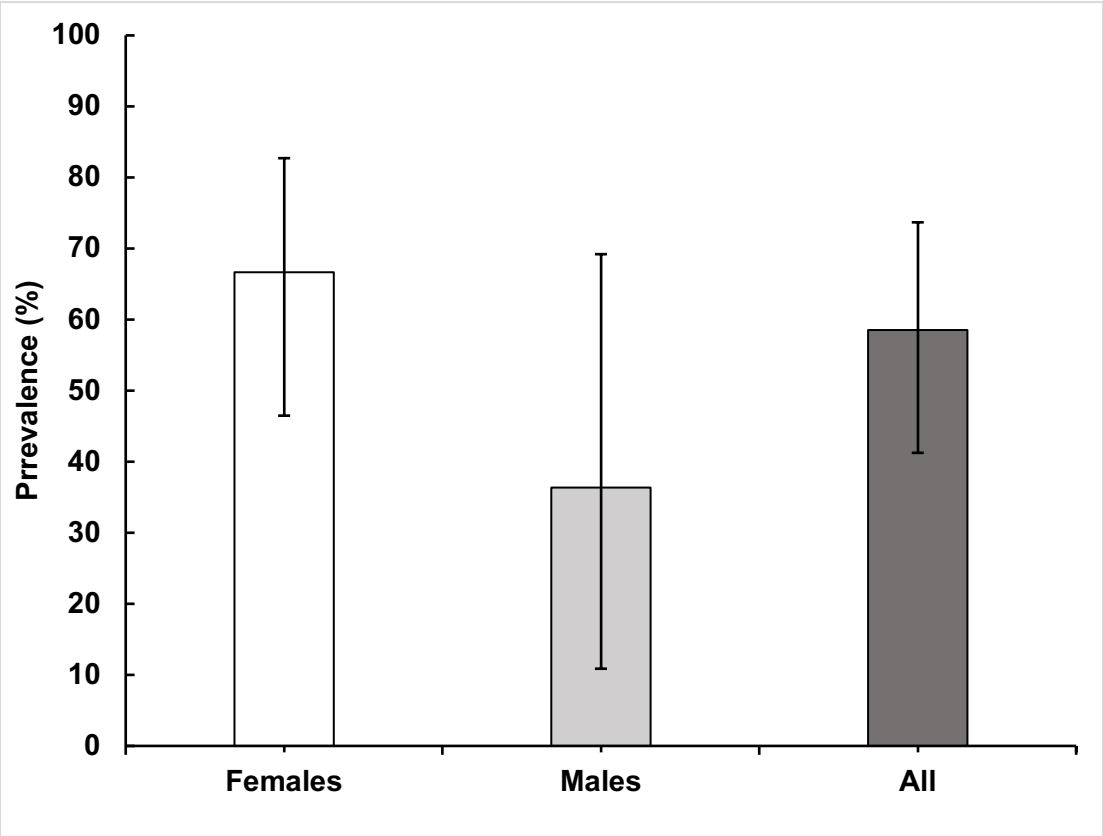

Figure 8. Prevalence (%) of *Nucleospora cyclopteri* (RNA detection) in lumpfish (n = 41) sampled in the present study. There was no significant difference between males (n = 11) and females (n = 30). Error bars for prevalence represent binomial 95 % confidence intervals.

3.4 Tissue tropism

Nucleospora cyclopteri RNA was present in all tissue types sampled in the present study. The variation in parasite density was very large, but the apparent pattern was that density was highest in the anterior kidney (Fig. 9), compared to other tissues. However, Ef 1a Ct-values varied significantly between the tissues (KW, $H_{(7,\ n=328)} = 71.5$, $P < 0.001$) (Fig. 10), potentially affecting density. Brain and blood results were therefore excluded from the statistical testing based on density. Statistical testing based on the other tissues in infected fish showed that the *N. cyclopteri* density varied (Friedman ANOVA $\chi^2_{(7,\ n=24)} = 61.6$, $P < 0.001$).

Post-hoc testing showed that muscle and liver had significantly ($P < 0.05$) lower density than most other tissues (Fig. 9). Among the other tissues, density in the anterior kidney was significantly higher than in the spleen, but different kidney parts did not differ significantly. These patterns were also seen in an analysis based on 'load'. In that analysis, brain was included, and *N. cyclopteri* load in the brain was together with muscle and liver lower than in

most other tissues. There was a significant positive correlation between parasite density of blood and density in blood rich tissues (kidney, gills, spleen, heart and liver) (Table 4). Conversely, there was no correlation with the density of parasites in brain and muscle with density of blood rich tissues or blood.

Fish with spore production (sporogony) could show a different tissue tropism than fish with only proliferative stages. Therefore, the observed patterns were also examined when disregarding fish with *N. cyclopteri* spores in the kidney (see section 3.7). This removed the three most heavily infected individuals, and changed the magnitude of the densities, but did not alter the observed pattern (Fig. 11).

Heart was the most often positive tissue and was positive in all fish sampled (Table 5).

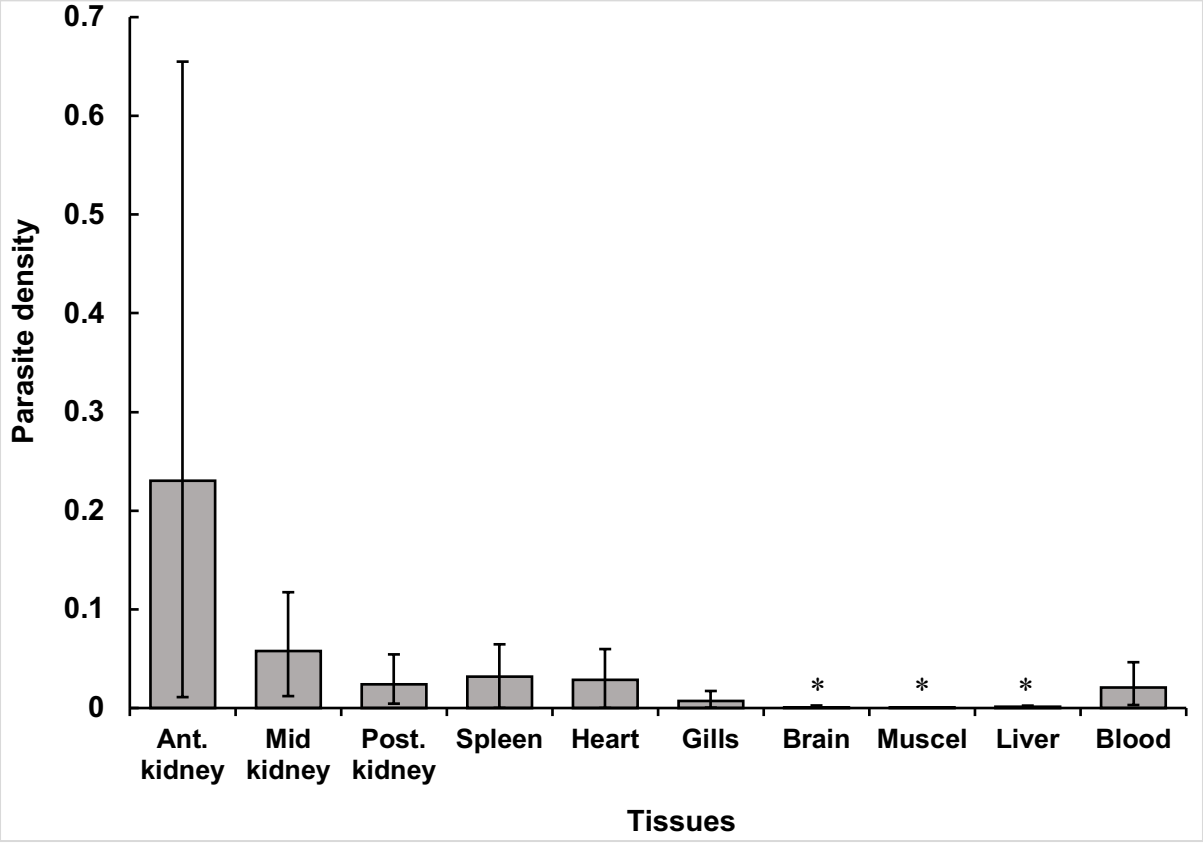

Figure 9. Densities of *Nucleospora cyclopteri* in samples from anterior kidney, mid kidney, posterior kidney, heart, gills, brain, muscle, liver and blood of infected lumpfish (n = 24) collected in the present study. Error bars for densities represent bootstrapped 95 % confidence intervals. Note that the densities may not be directly comparable across tissues, due to varying Ef 1a expression. Asterisks (*) indicate very low density, columns are present but not clearly visible. Abbreviations: Ant. Anterior, Post. Posterior.

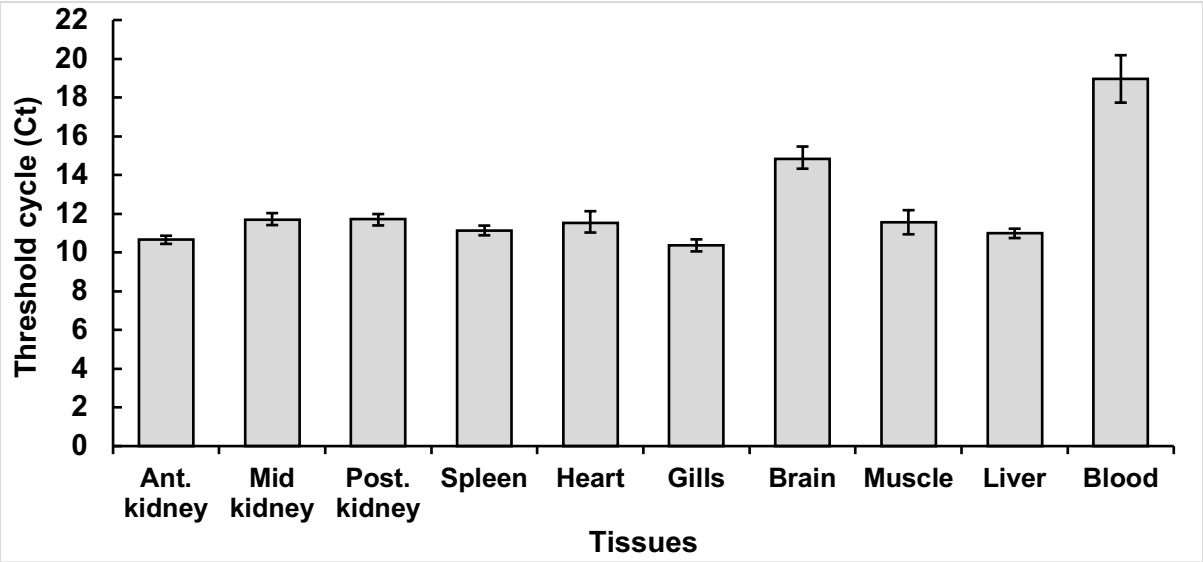

Figure 10. Mean Ct-values of lumpfish elongation factor 1a (Ef 1a) in samples from anterior kidney, mid kidney, posterior kidney, gills, spleen, heart, brain, muscle and liver of (next page) lumpfish (n=41) collected during the present study. Error bars for Ct-values represent bootstrapped 95 % confidence intervals. Abbreviations: Ant. Anterior, Post. Posterior.

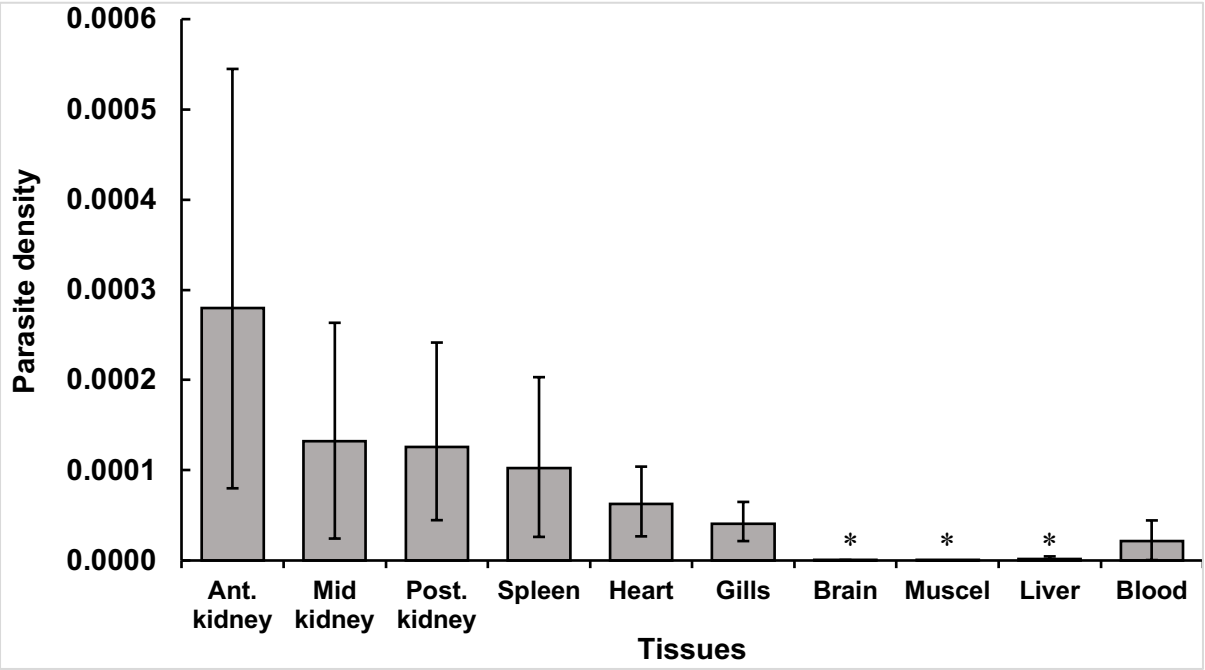

Figure 11. Densities of *Nucleospora cyclopteri* in samples from anterior kidney, mid kidney, posterior kidney, heart, gills, brain, muscle, liver and blood of infected lumpfish with no spores detected in microscopy (n = 21) (infections at pre-spore stage) (see sect. 3.7). Error bars for densities represent bootstrapped 95 % confidence intervals. Note that the densities may not be directly comparable across tissues due to varying Ef 1a expression. Asterisks (*) indicates very low density, columns are present but not clearly visible. Abbreviations: Ant. Anterior, Post. Posterior.

Table 4. Correlation matrix of *Nucleospora cyclopteri* densities between tissues of infected lumpfish (n=24). Spearman's rank correlation coefficients (r_s) lower left, significant levels (P) upper right. -, not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Significance levels were determined using Bonferroni corrections ($k = 45$).

	AK	MK	PK	Gills	Spleen	Heart	Brain	Muscle	Liver	Blood
AK		**	*	-	***	**	-	-	**	**
MK	**0.74**		**	-	-	**	-	-	-	**
PK	**0.65**	**0.73**		***	-	***	-	-	-	**
Gills	0.57	0.58	**0.86**		-	***	-	-	-	*
Spleen	**0.80**	0.62	0.55	0.51		-	-	-	*	***
Heart	**0.71**	**0.71**	**0.82**	**0.79**	0.55		-	-	-	**
Brain	0.27	0.14	0.17	0.33	0.40	0.16		-	-	-
Muscle	0.24	0.28	0.49	0.49	0.19	0.42	0.46		-	-
Liver	**0.75**	0.51	0.43	0.36	**0.68**	0.44	0.18	0.32		***
Blood	**0.75**	**0.75**	**0.74**	**0.66**	**0.78**	**0.69**	0.37	0.45	**0.77**	

Abbreviations: AK Anterior, MK mid kidney and PK Posterior kidney.

Table 5. Percentage of samples positive for *Nucleospora cyclopteri* RNA per tissue in infected lumpfish (n = 24).

Tissues	June sample (n=11)	November sample (n=13)	Total (n=24)
Anterior kidney	85	55	71
Mid kidney	62	55	58
Posterior kidney	62	91	75
Spleen	77	18	63
Heart	100	100	100
Gills	69	100	83
Brain	23	9	17
Muscle	15	9	13
Liver	62	18	42
Blood	31	9	21

Variation in densities among samples

Males were only available in the November sample and there were no significant differences in *N. cyclopteri* densities in anterior kidney between males and females in this sample (MW, $U_{11,19} = 70$, $P = 0.14$). The densities of the parasite in anterior kidney of females in June and November did not differ (MW, $U_{19,11} = 101$, $P = 0.90$). This result was also seen when analysing based on individual mean density.

3.5 Parasite developmental phase

The end product of the parasite development is spores inside the nuclei of leukocytes. Fish with such infections could display deviating parasite densities (tissue tropism) from those with only proliferative stages. When examining kidney imprints, leukocyte and blood smears, spores were seen in three of the 41 lumpfish sampled, representing 3/24 (13 %) of those infected. These were the lumpfish with the highest *N. cyclopteri* densities in the anterior kidneys. Spores were found in both kidney imprints, blood- and leukocyte smears, in cells with morphological characteristics of lymphocytes or lymphoblasts (Fig. 12). The spores were apparently only localized in the nuclei of the host cells.

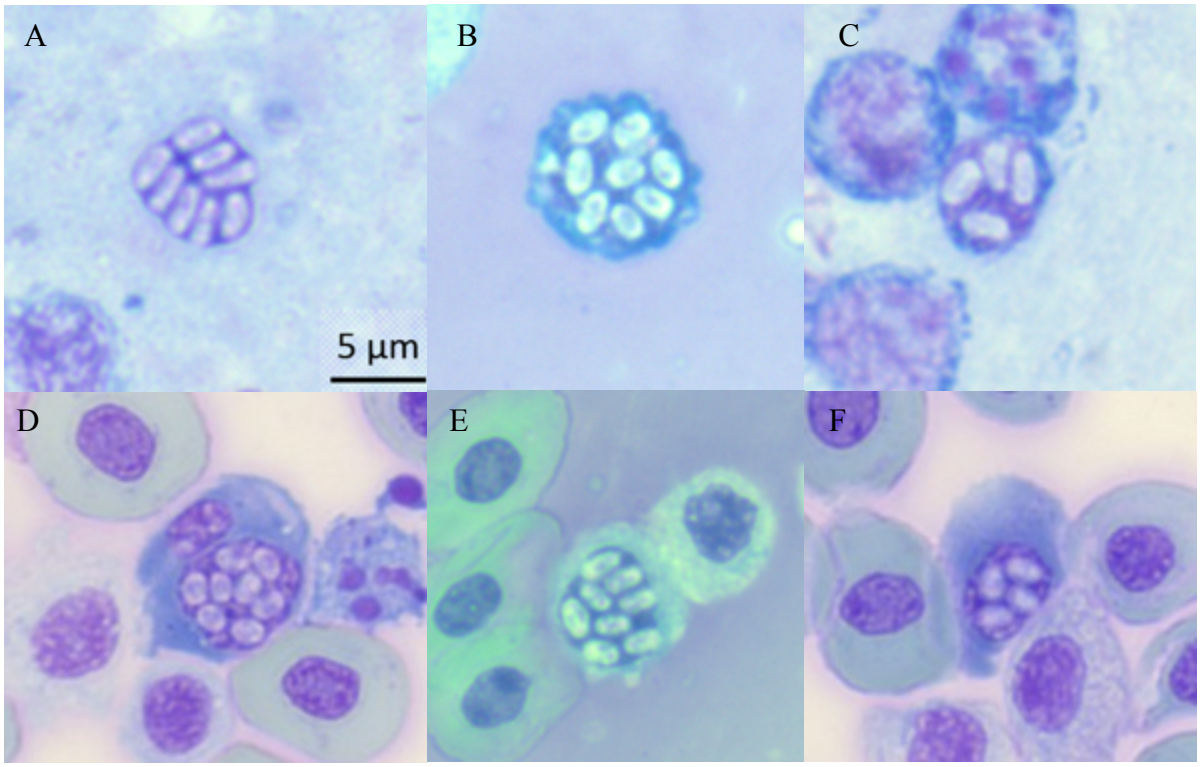

Figure 12. *Nucleospora cyclopteri* spores in nuclei; a free nucleus (**A**) or lymphocyte-like cells (**B-F**) in Hemacolor™ stained smears (**D, F**) and kidney imprints (**A-C, E**). Note the number of spores in a single nucleus, typically eight (**A, B, D, E**), but occasionally four (**C, F**). All images to the same scale as in (**A**). BF 1000 x.

There was an apparent pattern in the number of spores in the nuclei, generally being four or eight, the latter most common (76 %) (Fig. 13). The oval to elongate oval microsporidian spores measured 1.8 - 2.8 µm (min. - max.) (2.4 ± 0.3) (mean ± SD) in length and 1.0 – 1.8 µm (1.4 ± 0.2) in width (n=30). Length: width ratio was 1.2 – 2.7 µm (1.8 ± 0.4).

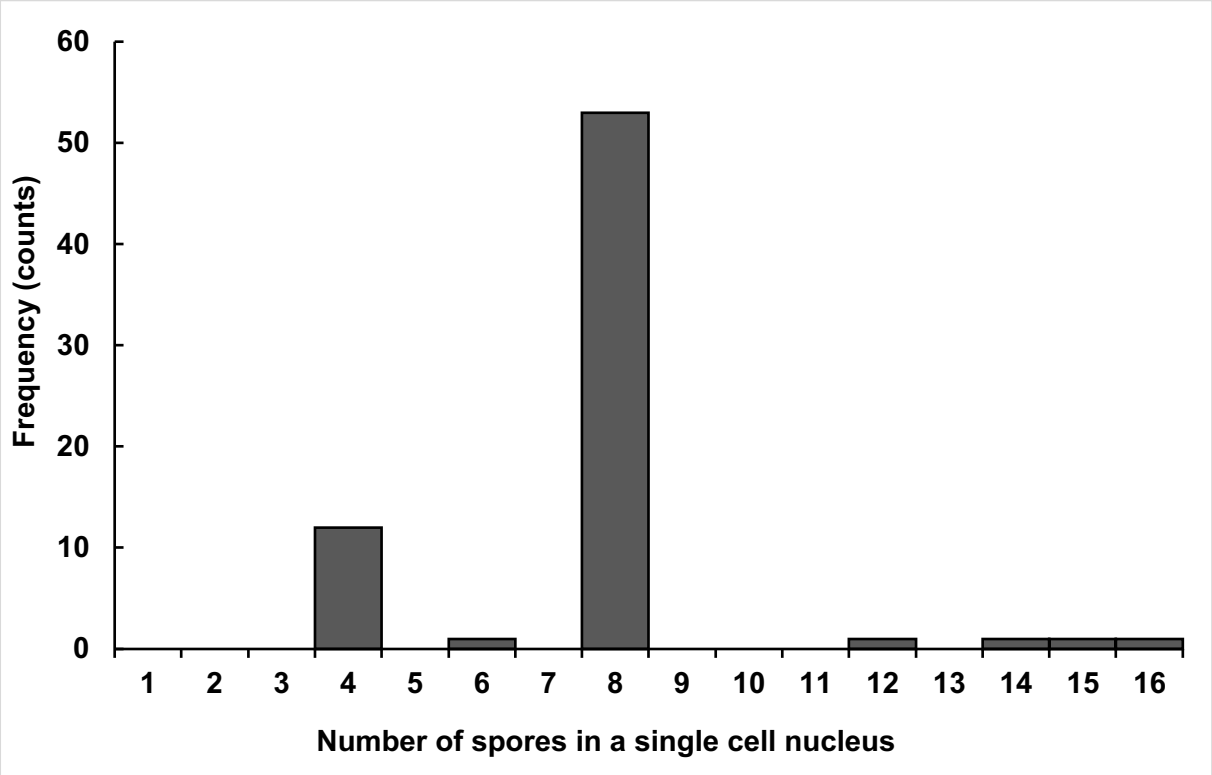

Figure 13. The numbers of *Nucleospora cyclopteri* spores observed in single cell nuclei, as the frequency (counts) of occurrence among 70 studied cells from 3 lumpfish.

3.6 Possible shedding

Bile and urine were positive for *N. cyclopteri* RNA. Individual mean density correlated significantly with parasite load in bile ($r_s = 0.45$, $n = 28$; $P < 0.05$) (Fig. 14) and urine ($r_s = 0.49$, $n = 36$; $P < 0.01$) (Fig. 15).

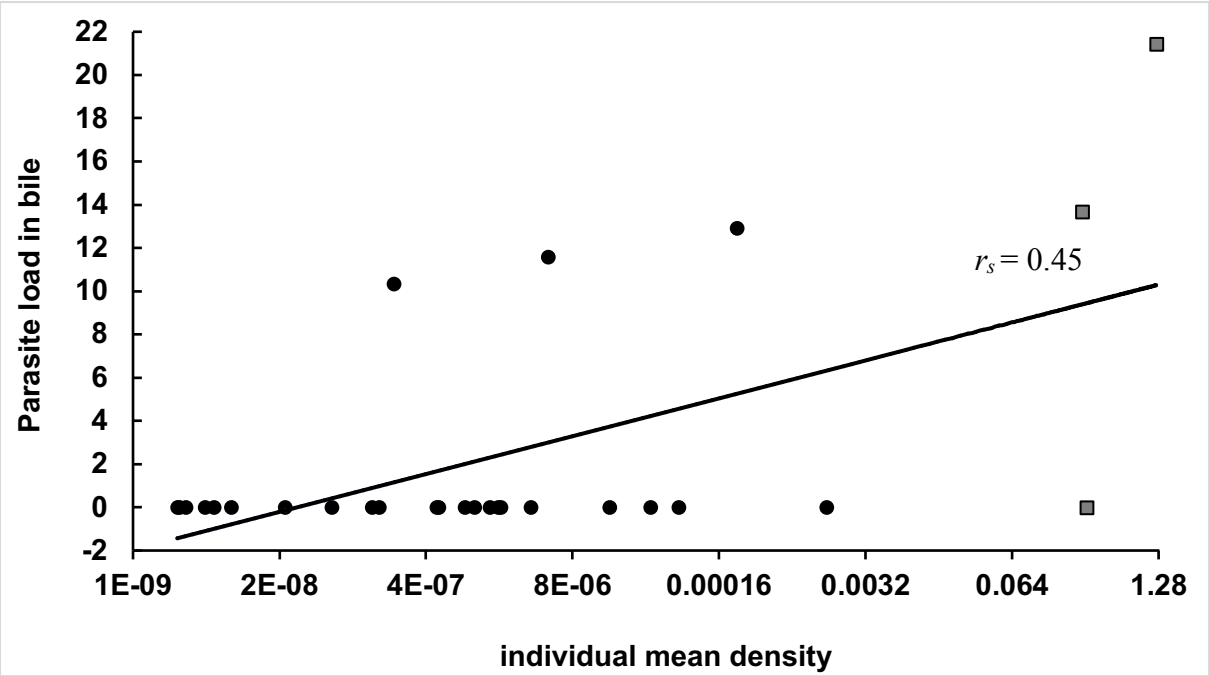

Figure 14. *Nuclospora cyclopteri* load in bile against individual mean density of lumpfish (n = 28) collected in the study. Trendline is logarithmic. Black dots represent lumpfish with no spores detected in microscopy (infections at pre-spore stage), and grey boxes represent fish with spores detected (see section 3.7).

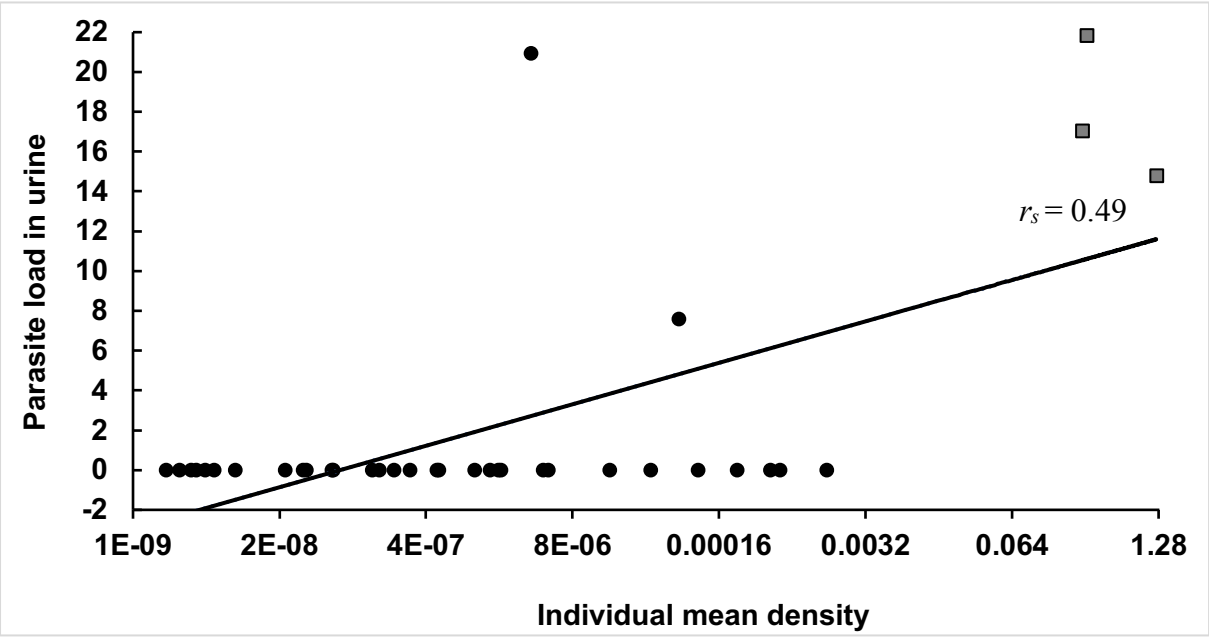

Figure 15. *Nuclospora cyclopteri* load in urine against individual mean density of lumpfish (n = 36) collected in the study. Trendline is logarithmic. Black dots represent lumpfish with no spores detected in microscopy (infections at pre-spore stage), and grey boxes represent fish with spores detected (see section 3.7).

***Nucleospora cyclopteri* in gonads**

Ovarian fluid samples (n = 39) and milt samples (n = 11) were taken and analysed by PatoGen Analyse AS. Three (8 %) of the ovarian fluid samples were positive for *N. cyclopteri* (Ct-values: 27-32). These three lumpfish were those with highest parasite densities in the anterior kidney and also showed the presence of spores in kidney or blood using microscopy. The milt samples were negative.

3.7 Assessment of non-lethal samples

3.7.1 Swabs

Swabs taken from the six different sites on lumpfish were positive for *Nucleospora cyclopteri* RNA. Swabs dipped in tank water were negative, however swabs of biofilm were positive (Ct: 30.1 – 30.3). The sucker revealed 83 % of infected fish, however this site also gave 82% false positives (Fig. 16). The swabbing sites with best detection ratio between true and false positives are opercular margin and behind the pectoral fin. Combining the two gives a detection proportion of 50 % true positives and 6 % false positives. All swabs were positive for Ef 1a and expression was best in dorsal skin when comparing the swabbing sites (Fig. 17).

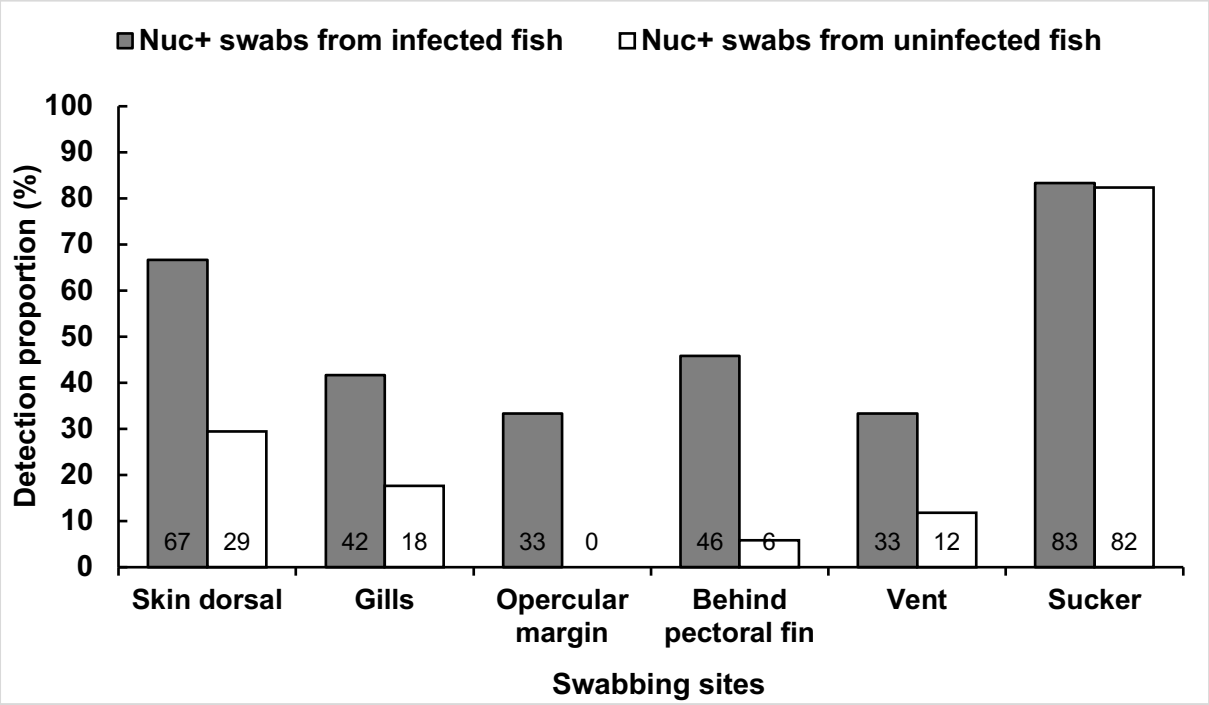

Figure 16. Detection proportion (%) of the swabbing sites to reveal *Nucleospora cyclopteri* infections in lumpfish (n=41). Abbreviations**: Nuc+** = positive for *Nucleospora cyclopteri* RNA.

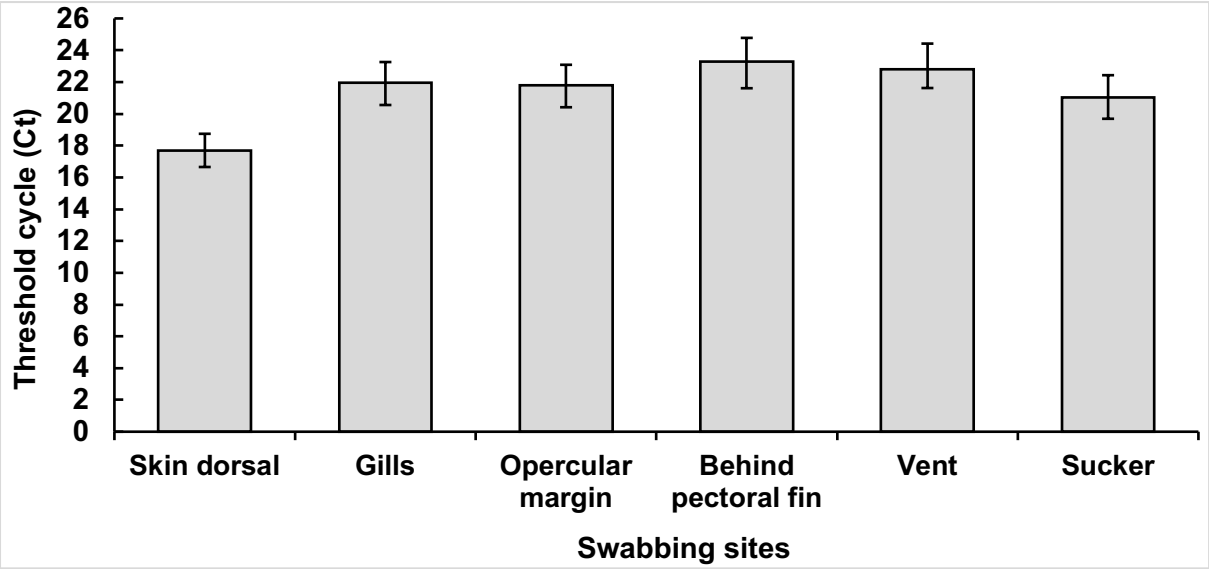

Figure 17. Mean Ct-values of lumpfish elongation factor 1a (Ef 1a) in swabs taken from lumpfish (n = 41) collected in the present study. Error bars for Ct-values represent bootstrapped 95 % confidence intervals.

3.7.2 Blood and gill samples

The three different biopsies taken in the present study were positive for *Nucleospora cyclopteri* RNA. Gill biopsies revealed the highest proportion of infected fish (83 %), however 47 percent of uninfected fish were detected as positive (Fig. 18). Blood and leukocytes gave no false positives and detected 25 and 42 percent of infected fish, respectively.

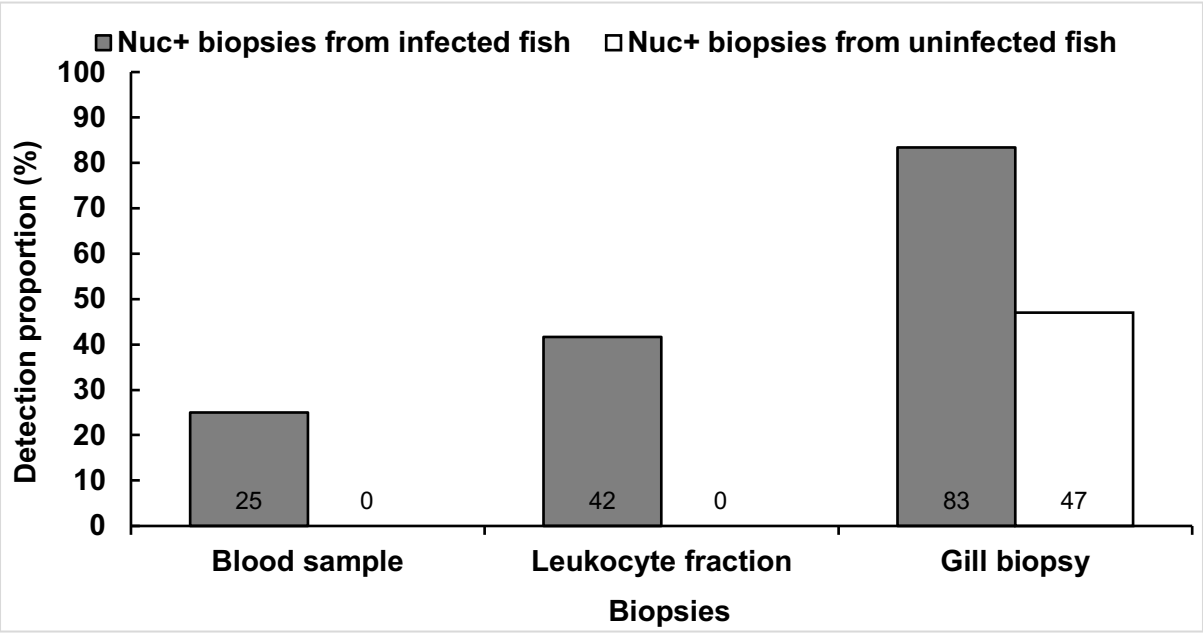

Figure 18. Detection proportion (%) of the biopsies to reveal *Nucleospora cyclopteri* infections in lumpfish (n = 41). Abbreviations: **Nuc+** = positive for *Nucleospora cyclopteri* RNA.

Correlation between leukocrit and *N. cyclopteri* density

There was a significant negative correlation (r_S = -0.42, n = 41; $P < 0.01$) between the leukocrit values and individual mean density of lumpfish in the present study (Fig. 19).

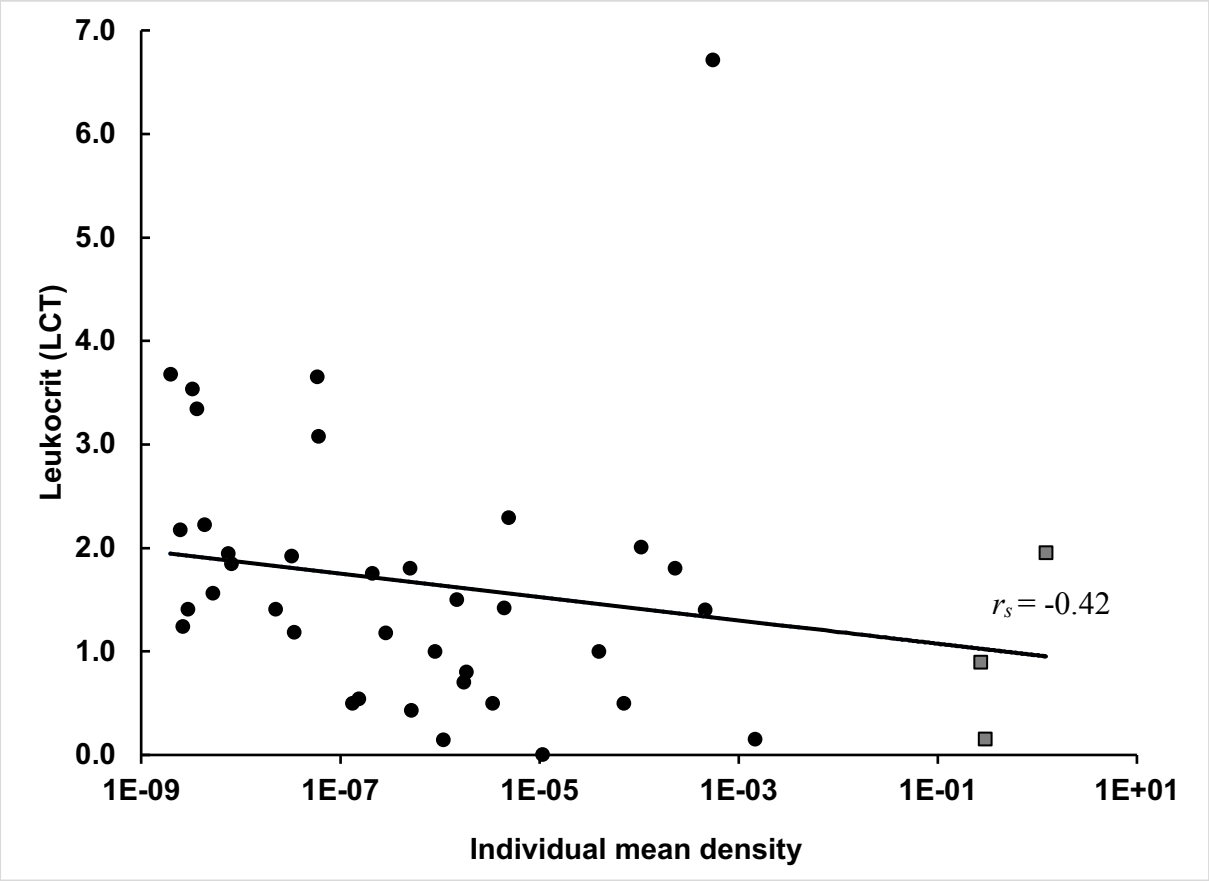

Figure 19. Leukocrit (LCT) against individual mean *Nucleospora cyclopteri* density. Trendline is logarithmic. Black dots represent lumpfish with no spores detected in microscopy (infections at pre-spore stage), and grey boxes represent fish with spores detected (see section 3.7).

4. Discussion

In the present study, wild-caught mature lumpfish were examined for infections with the microsporidian parasite *Nucleospora cyclopteri* using qPCR, in order to estimate parasite densities and reveal its tissue tropism. With an improved understanding of the tissue distribution of *N. cyclopteri*, the aim was also to reveal suitable non-lethal sampling methods for detection. The parasite was detected in all nine tissue types examined, but densities varied. Parasite densities were high in kidney, spleen, heart and gills of infected lumpfish. The parasite was detected using swabs on skin, gill and vent, and also blood samples and gill biopsies.

Macroscopical pathology

The collected lumpfish were caught by the same fishery that provides brood fish to supply egg and milt to many lumpfish producers in Norway, Ireland and Britain (Lein et al., 2017). All 41 fish studied appeared healthy and ready-to-spawn.

Swollen kidney (renomegaly) (Mullins et al., 1994; Freeman et al., 2013; Karlsbakk et al., 2014) and white nodules in kidney (Alarcon et al., 2016) have been previously reported in *N. cyclopteri* infected lumpfish. Of 77 wild-caught lumpfish in Iceland, 18 (23 %) showed kidney pathology (Freeman et al., 2013). Two of ten farmed lumpfish (20 %) had white nodules in the kidney (Alarcon et al., 2016).

In the present study 20 % of the fish showed macroscopic pathology in the kidney, in agreement with the above-mentioned reports. Mild renomegaly was observed in 5 fish and 3 fish had patchy pallor. However, no extensive enlargement of kidneys like those previously described was observed (Freeman et al., 2013; Karlsbakk et al., 2014).

Prevalence of infection

In the present study, detection limits were used when considering positive samples. It is common to use a detection limit when analysing using qPCR, due to several reasons for which the level of detection becomes problematic (Burns & Valdivia, 2008). This can be the presence of noise, unstable baseline, interferences to the signal, effect of analytical blanks, and losses during extraction, isolation or clean-up process (Burns & Valdivia, 2008). The choice of limit is important, since too low of a limit might risk eliminating valid results, or too high could increase false-positive results (Burns & Valdivia, 2008).

The prevalence used in the present study was based on two or more tissues among the kidney (anterior, mid or posterior), heart and spleen being positive. This approach is based on the assumption that when infected, the leukocyte target cells will spread systemically in the fish, so several samples should contain the parasite. Also, this restrictive approach mostly removes any impact from accidentally positive samples (cross contamination during sampling or analysis) when estimating the prevalence. These tissues were chosen based on previous studies using PCR (Freeman 2013; Alarcon 2016) and the results from the present tissue tropism study. Only results from internal organs could be included, due to the possible contamination from parasites in the water.

Freeman et al. (2013) reported a prevalence of 23 % of the 77 wild-caught lumpfish in Iceland, based on macroscopical signs. Alarcon et al. (2016) reported a prevalence of 50 % of the ten farmed lumpfish in sea pens using nested PCR.

The present prevalence of infection was 59 %. Our prevalence is higher than the Icelandic survey, but this was based only on macroscopic pathology. When examining 5 fish with and without macroscopic pathology using nested PCR, 5 and 4 were infected (Freeman et al., 2013), suggesting a high true prevalence. The prevalence reported from the farmed lumpfish in sea pens in Norway (Alarcon et al., 2016) are more similar to our findings.

Our results suggest that *N. cyclopteri* infections are common in wild-caught mature lumpfish in Norway.

Tissue tropism

Nucleospora cyclopteri has previously been detected in kidney, heart, spleen, skin, gills and eggs of a few lumpfish using nested PCR (Freeman et al., 2013; Alarcon et al., 2016), however nested PCR is not a quantitative method. To our knowledge, this is the first quantitative study of *Nucleospora cyclopteri* using qPCR. The following tissues were examined: anterior kidney, mid kidney, posterior kidney, spleen, heart, gills, brain (medulla oblongata), muscle, liver and blood. Because of the major pathological changes previously reported occurring in the kidney, and because the kidney in lumpfish is long and differentiated (Calderwood, 1891), both anterior, mid and posterior kidney were collected. Skin was not collected as a tissue piece since four different sites on the skin were swabbed. Liver was sampled due to pathology observed in this organ previously, in association with infections (Mullins et al., 1994; Alarcon et al., 2016). The related *N. salmonis* have also been detected in many tissues, including liver, blood and brain (Barlough et al., 1995). Sporogony of *N. cyclopteri* is previously observed in the nuclei

of lymphocytes (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013), however blood has never been used to detect the parasite.

In this study normalized expression (NE) was used as a measure of parasite density. However, a comparison of NE across tissue types relies on a similar expression of the internal reference gene, Ef 1a. This is often not the case, as shown in salmonids (Olsvik et al., 2005; Jørgensen et al., 2006) and zebrafish (McCurley & Callard, 2008), but variation tends to be within one or two Ct-values. There are currently no such studies of reference genes for lumpfish. In the present study, the Ct-values of Ef 1a did not vary much among most studied tissues (Fig. 10), except in brain and blood. In the case of blood, this was likely because the sampled blood volume did not correspond well to the tissue volumes sampled from blood-rich organs such as e.g. kidney and spleen. In brain however, there is a clearly lower Elf1a expression volume-by volume, than in the other tissues. The same has been found in other studies (Olsvik et al., 2005; Jørgensen et al., 2006; McCurley & Callard, 2008). Therefore, comparisons of NE did not include brain. The relatively stable Elf1a expression in the other tissues may reflect that a strict standardized size of tissue sample was attempted.

The densities of *N. cyclopteri* were highest in anterior kidney, followed by mid and posterior kidney, spleen, heart and gills. Parasite density was low in brain, liver and muscle. Analyses of various skin swabs showed that parasite densities were low in the skin.

The apparent wide tissue distribution of *N. cyclopteri* could be due to the presence of blood, since the parasite infects lymphocytes. However, parasite densities in tissues rich in blood (kidney, gills, spleen and heart) did not show very strong correlations with density in blood. The r_s^2 suggests that only 44-61 % of the high densities in these tissues could be explained by the presence of parasite in circulating blood (Table 4). *Nucleospora cyclopteri* was detected in only 25 % of the blood samples, and thus absent from the blood in 75 % of the infected fish. Parasite presence in circulating blood could be a limited to proliferative stages sufficient for a systemic spread, as proposed previously for *Nucleospora* spp. (Barlough et al., 1995; Alarcon et al., 2016), or parasitized cells with mature spores *en-route* to be shed. Therefore, the proliferation of leukocytes with pre-spore-stages could be confined to tissues showing high parasite densities. The high variation of parasite density in anterior kidney (Fig. 9) is believed to be due to proliferation of infected leukocytes in this tissue, as proposed previously by Karlsbakk et al. (2014). The patchy occurrence of the parasite seen in kidneys could support this. Similar proliferation and pathology are also reported in other species of the genus *Nucleospora* that targets nuclei of immune cells (Hedrick et al., 1990; Morrison et al., 1990; Nilsen et al., 1995). Anterior kidney in fish is known to contain a large lymphocyte population

and much of the immune response is induced and elaborated in this organ (Etlinger et al., 1976; Press, 1998; Press & Evensen, 1999). The extensive enlargements of the kidney in highly infected fish (Freeman et al., 2013; Karlsbakk et al., 2014), suggests that this site is important in regard to parasite intensity and possibly the density. Challenge experiment should be done to further clarify the tissue tropism of the parasite in the host.

The finding that heart is the most often positive tissue for the parasite is surprising (Table 5). This tissue was 100 % positive in infected fish in both samplings. The parasite density was also high in this tissue, supporting the importance of this site for parasite detection. An interesting observation is the differing proportion of positive samples between tissues in the June and November samples. Arguably, this could relate to different development phases of the parasite, or the course of infection in the fish. However, larger samples are needed to substantiate this, and further studies should be conducted to investigate the infection dynamics of *N. cyclopteri* in the host.

Parasite developmental phase

The end product of *N. cyclopteri* development is spores inside the nuclei of leucocytes, and spores have previously been revealed in kidney, spleen, gills, heart, liver, skin, intestine and ovary (Freeman et al., 2013; Alarcon et al., 2016). In the current study spores were seen in kidney imprints, leukocyte and blood smears, in three of the 41 lumpfish sampled. The highest parasite densities in the anterior kidney was seen in these fish. These findings indicate that at certain high densities, a subset of the parasite population may start sporogony in their host cells, while the rest likely continue proliferation (merogony).

The affected cells match previous descriptions (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013) as lymphoblast or lymphocyte cells. However, the size of spores is not consistent between the studies, likely due to the use of different methods. Measurements (mean length x width) of spores were 2.1 x 1.0 μm (Mullins et al., 1994) and 2.53 x 1.04 μm (Freeman & Kristmundsson, 2013) using TEM, and 3.1 x 1.3 μm measured in fresh imprints using microscope (Freeman et al., 2013). In the current study spores averaged 2.4 x 1.4 μm in air-dried smears and imprints. The differences in measurement are likely due to shrinkage during fixation or limitations of measuring small spores using a light microscope, as noted by Freeman and Kristmundsson (2013). Another source of variation in light microscopy could be various degrees of spore maturation. In this study a variation in length:

width ratio between 1.2 – 2.7 μm or sub-spherical to bacilliform spores is believed to be due to different levels of maturation.

The finding of a pattern with 4 or 8 spores formed inside host-cell nuclei represents new knowledge about the sporogony of *N. cyclopteri*. Previous descriptions were unclear on this, reporting 1-6 (Mullins et al., 1994), 1-14 (Freeman) and 3-8 (Alarcon) spores in a cell nucleus. Restricted space and resources in a single cell nucleus could be the reason for only eight spores being more common. Knowledge about the number of spores inside a cell nucleus can be used as an additional character for the identification of *N. cyclopteri.*

Shedding

The transmission mechanisms of *N. cyclopteri* between host fish is currently not known. Previous studies of the related *N. salmonis* shows that the life cycle likely involves horizontal and vertical transmission routes (Hedrick et al., 1990; Baxa-Antonio et al., 1992; Hedrick et al., 2012). *N. cyclopteri* is associated with severe pathological changes in the kidney (Mullins et al., 1994; Freeman et al., 2013; Karlsbakk et al., 2014; Alarcon et al., 2016) and some in liver (Mullins et al., 1994; Alarcon et al., 2016). It is therefore a possibility that spores are released from the host kidney via urine or from the liver via bile and gut. Spores of *N. cyclopteri* have also been found in close association with eggs, and vertical transmission has been suggested (Freeman et al., 2013).

The present analyses of bile and urine revealed *N. cyclopteri* to be present in both. Individual mean density correlated positively with parasite load in urine. These lumpfish also showed sporogony in the kidneys, so the parasite stage detected could be spores. Further investigations on heavily infected individuals may clarify this. Taken together the finding of positive bile, liver and vent swabs could indicate parasite shedding via gut. The role of bile in this, is not known.

Three (8 %) of the ovarian fluid samples were positive for *N. cyclopteri*. These results are consistent with previous findings of positive eggs and spores seen in ovary and near eggs (Freeman et al., 2013). Evidence for vertical transmission of *N. cyclopteri* is still lacking. However, research is being done on this topic as a part of the parent FHF project.

The results from swabbing of skin, gills and vent are also interesting regarding possible shedding. Higher *N. cyclopteri* densities were detected in swabs from infected individuals. This signal must be endogenous, since contamination from the water should be equal among infected and uninfected individuals in the tanks. Because the cells in skin would not normally

contain leukocytes or lymphocytes, this signal could also represent parasite shedding. In-situ hybridization or TEM studies could reveal the stages present.

Non-lethal detection

Non-lethal detection methods are needed to establish *N. cyclopteri*-free lumpfish brood stock. Especially if the breeding candidates are to be based on survivors from sea pens at the end of a production cycle. The methods should not be in violation of the animal welfare law, expose the fish to unnecessary strain or risk of inflicting damage to the fish (Dyrevelferdsloven, 2009). Swabbing is considered an easy and fast method of sampling, and there is minimal risk of inflicting damage to the fish or expose to unnecessary strain. Swabbing the pectoral fins and gills of Atlantic salmon can be used to detect ISAV infections (Aamelfot et al., 2015; Aamelfot et al., 2016). *Nucleospora cyclopteri* have previously been detected in skin, gills and intestine (Freeman et al., 2013; Alarcon et al., 2016). For this reason, swabbing of skin, gill and vent were selected. Swabbing the vent could detect parasites not only in the adjacent epithelium, but also from the gut, urine and in mature fish, the gonad. The different skin swabbing sites were chosen to represent diverse epidermal sites.

A problem with swabbing of fish kept in the same tank is the risk of contamination of the tank water by some heavily infected individuals. To account for this possibility, swab samples were taken from both tank water and biofilm on tank walls as controls. While swabs dipped in the tank water were negative for *N. cyclopteri,* swabs from biofilm were positive. The finding of positive urine and bile of some sampled lumpfish represents a possible source for this signal.

N. cyclopteri was detected in swabs from all six swabbing sites. The site revealing the highest proportion of infected fish was the sucker (83 %), however 82 % of swabs from uninfected fish were also positive. This is probably due to contamination of the sucker from the positive biofilm on the tank wall. The swabs from opercular margin and behind the pectoral fin were positive in a lower proportion of infected fish (33 and 46 % respectively), but few (0 and 6 %) swabs from uninfected fish were positive. However, combining the two swabs gives a better detection ratio (50 % true positives and 6 % false positives).

Still, swab testing performed in this manner cannot remove all infected fish. Keeping the fish individually in clean water prior to swabbing could reduce this problem, but this may be practically difficult.

Tissues suitable for non-lethal biopsy sampling must be accessible for sampling without any detrimental effects to the fish. *Nucleospora cyclopteri* has been detected in kidney, heart,

spleen and gills using PCR (Freeman et al., 2013; Alarcon et al., 2016), and in this study the parasite density was high in these tissues. Organs such as heart and spleen are unsuitable for non-lethal biopsy. Korsnes et al. (2009) demonstrated head-kidney as a suitable non-lethal biopsy from cod. However, this tissue is not readily accessible for sampling in lumpfish without any detrimental effects to the fish. The narrow opercular opening does not give sufficient access to the skin adjacent to the anterior kidneys. Gill tissues are however accessible for sampling and have been demonstrated as suitable non-lethal biopsies from salmonids to detect *N. salmonis* infections (Badil et al., 2011). Blood sampling of anesthetised fish is considered non-invasive and has been demonstrated as able to detect several pathogens in fish (Altinok et al., 2001; Lopez-Vazquez et al., 2006; Lopez-Jimena et al., 2010). Blood has not previously been used to detect *N. cyclopteri* using molecular methods, although spores have been revealed in the nuclei of lymphoblasts and lymphocytes (Mullins et al., 1994; Freeman et al., 2013; Freeman & Kristmundsson, 2013). Therefore, blood sampling is a potential method to detect the parasite in lumpfish, and especially the leukocyte fraction. Since *N. cyclopteri* stimulates an unusual proliferation of leukocytes in the host, elevated leukocrit values could be used to disclose infections. This is seen in the related *N. salmonis* where proliferation of infected lymphoblasts results in a leukemic condition in the host (Hedrick et al., 1990).

Gill biopsies were positive for the parasite in a high proportion of the infected fish, 83 %. However, biopsies from 47 % of uninfected fish were also positive, as could be expected since the gill biopsies are affected by the same contamination sources as the swabs.

Surprisingly a significant negative correlation was seen between the leukocrit values and individual mean density of lumpfish in the present study (Fig. 19). This further supports that proliferation of leukocytes with pre-spore-stages is confined to tissues with high parasite densities.

Positive whole blood samples were seen in only 25 % of the infected fish, and positive leukocyte fraction samples in 42 %. As could be expected, no samples were positive from uninfected fish, since the risk of cross contamination when taking blood samples is very low. The signal seen in the blood and leukocyte samples could stem from infected leukocytes, or free microsporidian spores. The crude blood separation used here could be much improved, and it is likely that analysis of blood samples could reveal a much higher fraction of the infected hosts. To refine this method, fresh blood samples should be brought to a lab on-ice, so improved separation of larger volumes could be done. Haugland et al. (2012) demonstrated the isolation of leukocytes from lumpfish using Percoll gradients, and this method might improve the separation.

Based on past experience, it was expected that individuals with pathology such as pronounced renomegaly should be found during the present study. Swabs and biopsies would likely detect a much higher proportion of infected fish if taken from apparently diseased fish like those reported by Freeman et al. (2013) and Karlsbakk et al. (2014). However, all fish studied in the present study appeared healthy. It is likely that lumpfish in sea pens would be more exposed to infection and also develop more intense infections because of stress and suboptimal rearing conditions. Further work on the non-lethal samples should be conducted on heavily infected lumpfish from sea pens.

Concluding remarks and future perspectives

In the present study, wild-caught mature lumpfish from a broodfish fishery were examined for the microsporidian parasite *Nucleospora cyclopteri.* This was done in order to reveal the tissue tropism of the parasite and to establish non-lethal sampling methods for detection. *Nucleospora cyclopteri* was detected in all nine tissues examined, and this supports that the infection may be systemic, as previously suggested. Parasite densities were highest in anterior kidney, followed by mid and posterior kidney, spleen, heart and gills. The highest variation of parasite densities was detected in the anterior kidney, and this might be related to more extensive parasite proliferation at this site. Challenge experiments with *N. cyclopteri* should be conducted to clarify the tissue tropism of the parasite in highly infected lumpfish.

It is also demonstrated that the parasite can be detected using skin, gill and vent swabs, blood samples and gill biopsies, and thus showing the possibility of non-lethal detection in lumpfish. The most promising non-lethal samples for detection were gill biopsies and leukocyte fractions from blood samples. Further work should be conducted in order to improve these detection methods further.

This effort will be important for establishing *N. cyclopteri*-free brood stock. The parasite may be vertically transmitted and have immunosuppressive effects on the host and could be important for reducing the mortalities of lumpfish in sea pens. This is essential for optimizing the use of cleaner fish with salmon and thus reducing the numbers of salmon louse in the sea.

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Appendix

Appendix 1 – RNA preservation solution recipe

400 ml of 0.5 M EDTA (74.44g disodium dihydrate per 400 ml pH to 8.0 with NaOH while stirring).
250 ml 1 M Sodium citrate (72.5g Sodium citrate trisodium salt dihydrate per 250ml.)
6 kg Ammonium sulphate
9350 ml H_2O stir on hot plate.
Adjust pH to 5.2 using conc. H_2SO_4

Appendix 2 – Fish data

Table 6. Lumpfish data. No. number: 101-111 from June sampling and 201-230 from November sampling. Length is measured in cm, weight in grams. F female and m male. Kidney appearance is normal if not noted otherwise. HCT hematocrit, ECT erythrocrit and LCT leukocrit.

no.	Length	Weight	Sex	Kidney appearance	HCT	ECT	LCT
101	43.0	1959	f		13.0	12.0	1.0
102	44.5	3015	f		22.0	22.0	0.0
103	47.0	2368	f		23.5	22.7	1.8
104	47.7	3330	f		17.0	15.5	1.5
105	42.8	2145	f		20.5	19.8	0.7
106	46.2	3474	f	Mild renomegaly	23.0	22.0	1.0
107	42.2	2360	f		20.0	19.5	0.5
108	41.2	2097	f		21.2	20.4	0.8
109	48.0	3603	f		22.0	19.5	0.5
110	41.0	1600	f		20.0	19.5	0.5
111	46.0	3580	f	Patchy pallor	17.0	16.1	0.9
201	46.0	2714	f		21.8	20.0	1.8
202	35.7	1728	m	Mild renomegaly	27.6	26.4	1.2
203	47.8	4623	f		19.5	15.8	3.7
204	47.6	3337	f		25.4	24.3	1.2
205	42.0	2280	f		23.8	21.6	2.2
206	46.5	3009	f	Patchy pallor	24.1	22.1	2.0
207	40.5	2301	f		19.8	18.0	1.8
208	36.2	1622	m	Patchy pallor	38.9	37.2	1.8
209	40.6	2209	f		22.7	21.1	1.6
210	48.0	5196	f		21.1	19.7	1.4
211	49.5	4116	f		24.4	23.0	1.4
212	47.2	2920	f		24.1	22.1	2.0
213	46.2	3790	f		21.3	20.1	1.2
214	45.5	3972	f		20.4	19.9	0.5
215	44.5	3586	f		17.7	15.5	2.2
216	38.3	1882	m		26.2	23.1	3.1
217	33.6	1221	m	Mild renomegaly	33.9	27.1	6.7
218	43.5	2317	f		24.8	21.2	3.6
219	40.7	2751	f		25.6	25.2	0.4
220	49.3	3921	f		33.2	31.8	1.4
221	41.8	2101	m	Mild renomegaly	32.3	30.5	1.4
222	36.1	1472	m		39.6	37.6	1.9
223	34.0	1335	m		36.6	34.4	2.3

Table 6. *Continued*

no.	Length	Weight	Sex	Kidney appearance	HCT	ECT	LCT
224	30.6	875	m		40.3	38.4	1.9
225	31.1	936	m		29.4	26.0	3.3
226	34.5	1618	m	Mild renomegaly	31.4	28.5	2.9
227	34.0	1494	m		32.3	28.8	3.5
228	44.5	2378	f		13.4	13.3	0.1
229	42.5	2567	f		20.0	19.8	0.2
230	42.0	2716	f		24.9	24.7	0.2

Appendix 3 – *Nucleospora cyclopteri* screening results

Table 7. *Nucleospora cyclopteri* screening results from tissues: anterior kidney, mid kidney, posterior kidney, gills, spleen, heart, brain, muscle and liver. Ct-values from Nuc and Ef 1a assay. No. number. ND not detected.

No.	Anterior kidney		Mid kidney		Posterior kidney		Gills		Spleen		Heart		Brain		Muscle		Liver	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
101	30.1	9.6	30.9	10.8	28.7	13.4	32.7	9.2	ND	10.1	27.8	15.6	ND	19.1	37.3	14.2	ND	11.2
102	31.0	10.4	25.2	10.3	29.8	12.8	24.2	11.1	ND	12.5	26.4	11.9	ND	16.1	ND	13.9	ND	12.4
103	35.9	11.2	30.2	10.3	32.9	12.6	33.1	9.9	ND	10.2	33.4	11.6	ND	16.5	ND	15.4	ND	9.9
104	33.9	9.8	29.0	10.5	31.7	9.8	25.0	10.2	ND	10.5	30.2	12.1	37.3	16.5	37.3	13.2	ND	11.4
105	36.7	10.6	31.2	12.5	37.4	10.8	35.4	11.4	ND	12.4	33.9	16.8	ND	14.6	ND	12.7	ND	10.5
106	35.0	9.7	37.4	9.9	29.9	11.4	32.1	10.9	ND	11.9	32.1	12.6	37.3	17.5	ND	13.9	ND	11.0
107	37.4	9.6	ND	14.2	27.4	11.4	27.1	13.6	ND	11.0	33.3	12.3	37.0	15.2	37.1	15.6	ND	11.5
108	ND	10.2	ND	10.4	31.9	12.0	25.8	10.9	ND	11.3	32.7	15.9	ND	17.6	ND	12.5	ND	11.0
109	23.0	10.1	ND	11.2	35.2	10.6	24.3	10.3	23.5	10.1	27.3	14.6	37.4	16.8	ND	13.0	ND	11.6
110	35.6	9.7	ND	14.1	32.2	9.7	30.6	10.7	37.4	10.7	33.0	11.5	ND	16.1	37.5	12.7	33.8	11.7
111	10.9	9.8	11.3	10.9	13.8	11.3	18.3	12.3	12.7	10.6	13.8	11.7	22.7	17.5	23.0	13.7	19.8	13.3
201	36.0	10.5	ND	11.8	ND	12.4	ND	9.4	37.7	10.7	ND	10.9	ND	12.6	ND	11.5	ND	10.6
202	31.9	11.5	36.1	12.6	37.3	11.8	33.9	9.4	33.8	11.8	33.0	10.4	ND	13.5	ND	10.2	37.8	10.8
203	38.0	10.9	ND	11.4	ND	12.1	36.9	11.4	ND	11.0	ND	10.4	ND	12.8	ND	13.4	37.8	10.8
204	37.2	11.4	34.9	11.8	ND	11.7	37.8	9.4	37.8	11.5	ND	10.6	ND	13.1	ND	10.1	34.5	10.2
205	37.2	11.2	ND	11.9	ND	12.4	26.5	9.3	ND	11.5	ND	10.7	ND	16.2	ND	10.2	ND	10.2
206	7.6	10.5	12.3	11.4	14.5	12.0	13.3	10.8	11.5	10.7	11.5	10.5	23.6	15.9	18.7	11.0	15.9	9.7
207	21.8	9.7	24.3	11.3	23.0	12.1	23.8	10.1	22.9	11.0	24.3	10.8	37.9	12.8	ND	10.6	31.1	11.0
208	31.5	11.2	ND	12.5	ND	11.6	33.3	9.7	36.5	11.0	34.6	10.7	34.2	15.7	ND	11.9	32.1	10.0
209	37.7	11.5	ND	11.9	ND	10.8	28.7	9.3	38.0	10.5	ND	10.0	ND	13.3	ND	9.2	ND	10.9

Table 7. *Continued.*

No.	Anterior kidney		Mid kidney		Posterior kidney		Gills		Spleen		Heart		Brain		Muscle		Liver	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
210	26.8	11.3	35.5	12.3	34.9	11.8	ND	10.0	36.0	12.0	33.6	10.5	ND	14.3	ND	10.9	34.8	10.1
211	20.2	10.3	23.3	11.9	23.6	13.0	22.8	9.5	24.3	12.4	23.1	10.8	ND	15.2	ND	11.1	37.1	10.6
212	23.1	10.2	24.7	11.5	24.2	11.7	24.4	10.0	25.6	11.3	26.4	12.6	36.6	15.2	ND	12.5	ND	10.5
213	ND	10.6	38.0	11.3	ND	10.9	ND	10.5	ND	9.7	ND	11.0	ND	13.6	ND	9.4	ND	9.9
214	36.4	11.6	35.1	12.2	ND	11.6	ND	9.9	33.6	11.4	32.4	10.5	37.1	17.4	ND	11.0	ND	11.5
215	ND	11.4	ND	12.9	ND	11.6	ND	9.9	37.8	11.0	ND	10.8	ND	13.4	ND	10.5	ND	10.7
216	38.0	11.0	ND	13.2	42.4	11.5	37.2	10.9	36.8	11.3	33.3	11.1	37.9	13.2	ND	11.9	ND	11.5
217	19.8	10.7	22.3	11.6	23.3	11.7	27.3	11.5	22.6	10.9	23.8	10.9	ND	13.4	ND	11.0	27.5	11.4
218	ND	9.5	37.0	11.5	ND	11.6	ND	9.2	33.8	10.9	34.2	10.6	35.9	13.5	ND	9.0	ND	11.2
219	35.6	11.1	36.9	12.3	37.8	11.1	ND	10.6	30.4	11.6	ND	11.0	ND	13.7	ND	9.2	ND	11.1
220	ND	10.9	35.2	11.8	ND	11.5	26.1	10.0	ND	11.1	ND	10.9	ND	14.2	ND	9.7	ND	12.0
221	ND	11.1	37.0	10.5	ND	12.4	ND	9.7	ND	10.4	ND	11.3	ND	12.7	ND	10.9	ND	11.5
222	37.7	10.8	ND	11.8	ND	12.4	ND	10.7	ND	11.4	35.1	9.4	ND	14.0	ND	12.3	ND	11.2
223	26.2	10.3	35.5	12.0	ND	12.7	ND	10.8	ND	11.4	27.9	10.4	ND	14.2	ND	12.2	27.0	11.6
224	ND	12.1	ND	10.7	ND	12.4	37.5	10.3	ND	11.2	34.0	11.1	ND	16.8	ND	9.3	ND	10.7
225	ND	10.6	38.3	12.1	ND	11.1	ND	9.6	ND	10.5	ND	11.1	ND	13.2	ND	10.4	ND	11.3
226	ND	10.3	ND	12.1	ND	11.9	ND	10.0	ND	10.6	ND	11.3	ND	14.6	ND	11.1	ND	11.4
227	ND	10.5	37.9	11.6	ND	11.0	35.1	10.9	ND	10.4	ND	11.7	ND	12.5	ND	10.6	ND	10.9
228	28.4	10.7	36.6	11.7	ND	13.4	ND	10.9	37.1	11.4	ND	11.6	ND	15.5	ND	9.7	ND	10.8
229	20.0	11.8	20.5	11.5	20.8	11.1	22.6	10.8	21.1	11.6	22.2	11.3	ND	15.1	ND	12.5	29.9	10.3
230	11.9	10.9	11.9	11.4	13.2	12.1	14.7	10.3	14.9	12.9	13.4	11.1	23.7	13.1	19.2	9.2	17.6	10.9

Table 8. *Nucleospora cyclopteri* screening results from bile, urine, blood and leukocyte fraction. Ct-values from Nuc and Ef 1a assay. No. number. ND not detected. ns not sampled.

No.	Bile		Urine		Blood		Leukocyte fraction	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
101	ND	13.8	ND	28.0	ND	18.4	ND	18.4
102	ND	26.7	ND	22.7	ND	19.7	ND	25.2
103	ND	22.6	ND	36.1	ND	23.8	ND	18.8
104	ND	28.2	ND	31.5	ND	13.2	ND	16.1
105	ND	21.8	ND	28.4	ND	13.6	ND	18.4
106	ND	19.3	ns	ns	ND	16.5	ND	20.8
107	ND	23.2	24,044	18.7	ND	16.4	ND	18.2
108	ND	28.5	ND	19.3	ND	14.1	ND	19.6
109	ND	22.9	37,404	20.6	ND	18.1	28,823	17.5
110	ND	21.3	ND	17.2	ND	17.9	ND	17.5
111	31,32	23.7	27,961	20.5	18.9	16.1	18,437	18.8
201	ns	ns	ND	33.0	ND	12.1	ND	17.6
202	ns	ns	ND	32.9	ND	12.2	ND	18.7
203	ns	ns	ND	26.1	ND	19.3	ND	18.1
204	ns	ns	ND	28.6	ND	20.9	ND	20.1
205	ND	31.4	ND	24.2	ND	19.1	ND	19.4
206	23,552	27.8	30,203	28.0	25.0	23.7	14,36416	14.2
207	32,083	23.8	ND	30.5	30.7	18.5	37,50073	21.6
208	34,663	20.0	ND	28.9	ND	14.9	ND	17.2
209	ND	28.5	ND	30.3	ND	21.5	ND	23.8
210	ns	ns	ND	32.2	ND	22.6	33,0952	16.4

Table 8. *Continued.*

No.	Bile		Urine		Blood		Leukocyte fraction	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
211	ns	ns	ND	34.0	ND	23.1	26,12095	14.2
212	ns	ns	ND	28.0	ND	23.8	30,239	16.7
213	ND	35.4	ND	30.1	ND	24.8	ND	15.3
214	ND	30.2	ND	28.7	ND	23.7	ND	13.6
215	ND	31.0	ns	ns	ND	22.7	ND	16.0
216	ns	ns	ND	30.5	ND	16.8	ND	17.7
217	ns	ns	ND	20.8	31.2	15.1	28,76446	15.7
218	ND	27.9	ND	29.6	ND	21.6	ND	16.5
219	ND	21.9	ND	24.6	ND	19.1	ND	15.3
220	ND	32.6	ND	23.5	ND	24.3	ND	21.9
221	ND	30.6	ns	ns	ND	16.8	ND	18.1
222	ND	28.1	ns	ns	ND	14.7	ND	14.7
223	33,415	25.1	ND	28.0	ND	17.8	ND	15.2
224	ns	ns	ND	24.6	ND	22.5	ND	12.5
225	ns	ns	ND	27.8	ND	17.7	ND	11.7
226	ns	ns	ND	23.8	ND	16.9	ND	13.8
227	ns	ns	ND	24.3	ND	14.6	ND	16.5
228	ND	36.8	ND	21.2	ND	21.1	ND	18.4
229	ND	34.4	ND	23.5	35.6	24.5	28,254	18.5
230	ND	31.3	23,14503	19.9	24.4	23.2	13,22	12.3

Table 9. *Nucleospora cyclopteri* screening results from swabs on different sites: skin dorsal, gills, opercular margin, behind pectoral, vent and sucker. Ct-values from Nuc and Ef 1a assay. No. number. ND not detected.

No.	Skin dorsal		Gills		Opercular margin		Behind pectoral		Vent		Sucker	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
101	ND	16.4	ND	28.1	ND	23.3	ND	18.9	ND	27.6	ND	25.5
102	37.3	18.7	ND	28.6	ND	22.7	37.4	16.6	ND	18.7	34.0	23.9
103	ND	17.0	ND	27.3	ND	17.8	ND	19.2	ND	27.6	ND	33.6
104	38.0	16.1	ND	28.3	ND	21.2	ND	21.2	ND	27.3	ND	22.8
105	34.5	14.8	ND	23.6	ND	18.0	ND	19.1	ND	24.7	ND	25.2
106	30.0	14.7	ND	21.0	34.7	18.1	30.6	14.4	37.5	19.1	31.0	24.7
107	30.2	18.6	37.8	25.0	ND	24.5	33.3	15.9	28.4	22.8	33.2	24.9
108	30.7	16.8	33.8	19.8	33.8	17.2	35.3	16.8	42.9	19.4	34.4	25.7
109	28.4	15.4	34.8	22.9	34.2	17.4	33.5	16.5	ND	22.5	31.7	22.1
110	28.0	14.3	38.1	23.3	34.2	16.6	34.2	15.3	ND	27.2	30.7	19.4
111	29.0	15.1	29.3	23.4	30.7	19.3	33.8	19.8	36.0	31.3	35.1	27.2
201	36.5	15.2	ND	30.5	ND	34.7	ND	30.9	ND	18.3	35.7	18.1
202	32.8	19.3	35.6	23.4	ND	22.5	ND	20.5	ND	18.7	34.6	23.9
203	ND	16.9	ND	28.4	ND	29.6	ND	25.2	ND	28.2	ND	28.3
204	32.6	15.2	37.0	18.6	ND	21.1	ND	34.3	ND	21.2	29.5	16.5
205	ND	15.2	34.7	18.7	ND	23.1	37.6	17.2	37.4	19.9	30.6	18.1
206	23.1	16.1	24.4	21.2	34.6	23.0	28.8	21.8	22.3	19.2	31.9	21.8
207	35.4	22.9	36.1	17.3	ND	24.9	37.8	24.6	29.1	17.4	29.6	17.0
208	ND	24.0	ND	31.2	ND	23.6	ND	28.1	ND	16.5	30.7	17.0
209	ND	17.1	ND	25.2	ND	21.3	ND	29.3	ND	21.9	31.8	16.9
210	ND	22.9	ND	25.1	ND	17.5	35.0	20.2	ND	21.8	28.3	16.1

Table 4. *Continued.*

No.	Skin dorsal		Gills		Opercular margin		Behind pectoral		Vent		Sucker	
	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a	Nuc	Ef 1a
211	31.0	15.5	ND	19.9	ND	14.6	ND	28.3	ND	28.7	27.6	18.5
212	ND	15.5	37.6	19.8	38.2	15.8	ND	24.6	ND	26.1	26.3	16.6
213	ND	18.8	ND	21.5	ND	20.7	ND	26.3	ND	24.2	29.9	17.5
214	ND	16.6	ND	19.0	ND	17.4	ND	20.3	ND	16.7	36.8	20.5
215	27.8	16.8	ND	21.1	ND	17.9	ND	23.7	35.5	18.4	31.6	19.9
216	ND	18.7	ND	18.1	ND	22.5	ND	29.0	ND	26.4	30.9	22.7
217	32.8	15.0	ND	17.2	ND	22.1	ND	26.6	ND	25.6	35.6	18.6
218	30.9	14.2	ND	25.5	ND	21.5	ND	26.8	ND	23.1	33.4	16.1
219	ND	20.4	ND	19.5	ND	28.1	ND	31.4	ND	24.6	34.5	19.4
220	37.6	16.7	26.0	13.5	ND	21.3	ND	20.6	ND	20.7	34.8	20.6
221	ND	18.3	ND	18.4	ND	20.9	ND	24.9	ND	25.4	ND	23.5
222	37.2	23.0	ND	23.7	ND	26.3	ND	22.0	ND	17.0	32.6	18.4
223	ND	17.1	ND	21.0	ND	21.6	ND	27.8	ND	19.7	28.4	19.7
224	ND	25.3	ND	20.5	ND	28.9	ND	26.5	ND	17.9	30.1	21.5
225	ND	16.6	ND	14.6	ND	26.9	ND	28.0	ND	20.6	ND	19.9
226	ND	19.6	ND	19.8	ND	19.8	ND	23.2	ND	30.4	34.9	21.8
227	ND	19.4	ND	19.4	ND	25.4	ND	26.5	ND	27.0	32.0	20.3
228	ND	15.5	ND	15.5	ND	21.0	ND	23.2	ND	24.4	34.4	19.7
229	ND	20.8	ND	19.0	ND	23.8	ND	25.3	34.5	22.0	36.2	21.5
230	25.8	17.8	33.4	22.6	30.7	19.4	36.5	24.7	25.4	24.4	27.7	16.9