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A novel qPCR assay for the rapid detection and quantification of the lumpfish (*Cyclopterus lumpus*) microsporidian parasite *Nucleospora cyclopteri*

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ABSTRACT

Nucleospora cyclopteri is an intracellular fungal-related parasite that causes microsporidiosis in Atlantic lumpfish (*Cyclopterus lumpus*), a commercially important species widely used to control sea lice in salmon farming. The parasite causes important economic losses in lumpfish aquaculture, but there is little information on its prevalence or pathogenesis. We compared the sensitivity and efficiency of traditional screening methods using macroscopic and microscopic techniques, with a nested PCR and a newly developed qPCR assay. We also examined the distribution of the parasite in different tissues and quantified parasite loads in fish with and without macroscopic symptoms. Our results indicate that 93.3% of the farmed lumpfish we sampled were infected with *N. cyclopteri,* including 46% asymptomatic fish without any clinical signs of infection. Asymptomatic fish had much lower parasite loads, quantified using qPCR. The infection was detectable in all tissues, including blood, consistent with systemic infection. While both nested PCR and qPCR assays were more sensitive than traditional screening methods, only the qPCR assay provides a quantitative assessment of parasite loads, which should prove useful for managing microsporidial outbreaks in lumpfish aquaculture.

1. Introduction

Microsporidia are a group of common unicellular fungal-related parasites of insects, fish and mammals which disperse via spores (Capella-Gutiérrez et al., 2012; Dunn and Smith, 2001; Katinka et al., 2001; Keeling, 2014; Metenier and Vivares, 2001) and have a relatively simple life cycle, consisting of three primary developmental stages: an infective or spore-phase, an intracellular proliferative phase, and a spore-forming phase (Cali and Takvorian, 2014). Being obligate intracellular parasites, microsporidia generally proliferate by exploiting the apoptotic mechanisms of host cells (Martin-Hernandez et al., 2017), causing histological tissue degeneration, chronic and debilitating diseases and, in some cases, host mortality (Dussaubat et al., 2012). Microsporidia are known to cause production losses in animal farming, including aquaculture (Frenette et al., 2020; Stentiford et al., 2016). In aquatic systems the main route of transmission seems to be direct contact with infected individuals, and most microsporidian, such as *N. salmonis*, infect fish without the involvement of intermediate vectors or hosts (Lom and Nilsen, 2003). Different species of microsporidia display a different range of tissue tropism. For instance, mammalian microsporidian *Encephalitozoon cuniculi* affects the renal and nervous systems in rabbits, honey bee microsporidian *Nosema ceranae* mainly infects midgut tissues and *Spraguea* targets the nervous system of anglerfish (Freeman et al., 2011; Frenette et al., 2020; Huang and Solter, 2013; Rodriguez-Tovar et al., 2016). Most microsporidia reside in the cytoplasm of host cells, but a few species are known to infect the nucleoplasm of host cells such as *N. salmonis* and *Microsporidium rhabdophilia* from *Oncorhynchus sp.* (Baxa-Antonio et al., 1992; Chilmonczyk et al., 1991; Modin, 1981), *N. secunda* from *Nothobranchius rubripinnis* (Lom, 2002), *N. braziliensis* from Nile tilapia, *Oreochromis niloticus* (Rodrigues et al., 2017) and *N. cyclopteri* from lumpfish (Mullins et al., 1994) (Freeman et al., 2013).

Lumpfish (*Cyclopterus lumpus*) is a commercially important species in aquaculture, used in the salmon industry as a biological method to remove sea lice (*Lepeophtheirus salmonis*) (Powell et al., 2018). Lumpfish are susceptible to microsporidian infections including *Tetramicra brevifilum* (Scholz et al., 2017) and *Nucleospora cyclopteri*, which is closely related to *N. salmonis* and causes tissue damage and mortalities with important economic and welfare consequences (Freeman et al., 2013). *N. cyclopteri* has been identified in farmed lumpfish from Eastern Canada, Norway and the UK and also in wild lumpfish from Iceland, Norway and the UK, resulting in irreversible histological changes, kidney pathologies and high mortalities (Alarcon et al., 2016; Freeman et al., 2013; Mullins et al., 1994). *N. cyclopteri* infection appears to disrupt normal swimming and causes loss of weight in lumpfish before progressing to more advanced clinical signs of infection and mortality

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(Alarcon et al., 2016). Based on signs of infection, including macroscopic enlargement of the kidney (renomegaly), the prevalence of clinical microsporidiosis in Icelandic wild lumpfish was estimated to be 23%, although nested PCR analyses of a subsample suggested a potentially higher rate of infection (Freeman et al., 2013). There is no effective treatment for *N. cyclopteri* infection in lumpfish and the prevalence is expected to be high, with potentially higher parasite loads in fish with more obvious clinical signs of infection (Ombrouck et al., 1997; Powell et al., 2018; Stentiford et al., 2016). Similar to other microsporidial species, *N. cyclopteri* produce infectious spores characterized by a thick chitinous outer layer and an internal coiled polar-tube used during the invasion of host-cells (Freeman and Kristmundsson, 2013; Vavra and Lukes, 2013). Many spores can be produced from a single infected cell, facilitating its systemic dispersion (Freeman and Kristmundsson, 2013). Yet, for *N. cyclopteri* the life cycle and patterns of transmission are still largely unknown (Warland, 2018).

The most widely used methods of diagnosis for microsporidia infection are macroscopic examination, histological staining and, more recently, conventional PCR. Macro and microscopic examination are time-consuming, require histological expertise, and suffer from several shortcomings, including low sensitivity and poor identification at species level. In addition, microscopic staining is not easily applied to the screening of eggs or larval fish, nor does it reliably detect the pre-sporogonic parasite stages. In contrast, conventional PCR tends to have higher sensitivity (Freeman et al., 2013), but it does not provide a quantification of parasite loads. Here we compared the sensitivity and efficiency of conventional PCR, nested PCR and a novel real-time qPCR assay with macrospopic and microscopic screening of *N. cyclopteri* in different tissues of farmed lumpfish of different origins and degrees of signs of pathology.

2. Methods and materials

2.1. Collection and preparation of samples

A total of 60 farmed lumpfish, including 43 adults (age: 13–24 months; weight range: 140–1946 g, weight average: 462 g, SD: 350.92) and 17 juveniles (age: 6–9 months; weight range: 13–18.3 g, weight average: 15.15 g, SD: 2.34) were examined for microsporidia infection at two recirculation aquaculture facilities in the UK between December 2016 and July 2017. The forty-three adults (28 males, 15 females) originated from parents from three distinct geographical origins (Iceland $n = 5$; Norway $n = 1$, Britain $n = 37$) and were randomly sampled at Farm 1, along with kidney samples from 7 juveniles from Britain that had clear clinical signs of infection by *N. cyclopteri* and that were used as positive controls. Ten juveniles with no previous history of microsporidia infection from Iceland ($n = 5$) and Norway (n = 5) were sampled at Farm 2. Fish were humanely euthanized in accordance to Home Office Schedule 1, and weight (g) and sex were recorded.

Small samples of kidney, spleen, heart, liver, gill, gonads and blood were collected with sterile tools, from the same locations when possible, although not all tissues could be collected for all fish (sample size range = 19–53 individuals). To avoid cross-contamination, new surgical blades were used between samples and forceps were washed with bleach, rinsed, and dipped in 95% ethanol. Tissues were kept in absolute ethanol at 4 °C before DNA extraction. Peripheral blood was withdrawn aseptically from the caudal vein immediately post-euthanizing using sterile 23-gauge blue sterile needles with disposable 1-ml syringe (Terumo), and was immediately placed in 2 ml ethylene-diaminetetraacetic acid (EDTA) anticoagulant tubes. Samples were stored at −20 °C prior to DNA extraction.

Kidney samples of 7 juveniles with clinical signs of microsporidiosis were fixed in 10% buffered formalin and were submitted to the Institute of Aquaculture Diagnostics Unit, University of Stirling (UK) for histopathological examination (Case 160,078).

2.2. Pathological examination

Pathological manifestations of microsporidiosis were assessed based primarily on changes in the head kidney as reported by Mullins et al. (1994). A smooth and evenly red-coloured kidney was considered normal, whereas the presence of patchy pallor and various degrees of renomegaly were considered to be signs of microsporidiosis. Other non-specific clinical signs of microsporidiosis, such as bilateral exophthalmia, ascites and skin lesions were also recorded. Kidney smears of 12 juveniles were stained with Diff-Quik® (Speedy-Diff Kit, Clin-Tech Limited, UK) to check for the presence of microsporidial spores, as part of a presumptive diagnosis (Joseph et al., 2006).

2.3. DNA extraction from tissues and blood

DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions using ~25 mg of tissue (~2 mm cube). Gonads were rinsed with phosphate buffer saline pH 7.4 (Gibco™ ThermoFisher Scientific) before extraction. For peripheral blood, 10 µl of whole blood collected in tubes with EDTA were briefly vortexed and diluted to a 220 µl final volume with phosphate buffer saline, pH 7.4 before DNA extraction. An extraction blank was carried out for every 25 samples. Extraction was done separately between clinically infected and non-infected groups based on macroscopic examination. DNA was extracted from 239 samples (53 kidney, 33 spleen, 32 liver, 33 heart, 43 gill, 26 gonad, and 19 blood samples; see Table 1 for details). Extracted DNA was stored at −20 °C prior to downstream analyses. DNA concentration and quality was assessed using a NanoDrop™ 2000c Spectrophotometer (ThermoFisher Scientific).

2.4. Polymerase chain reaction (PCR) assay

We used the nested PCR protocol described in Freeman et al. (2013). It included two rounds of amplification, with amplicons from the first reaction being used as templates for the 2nd PCR. The first pair of *Nucleospora* semi-specific primers (LN1_fwd 5′ atcctaggatcaaggacgaag and LN1_rev 5′ aatgatatgcttaagttcagg, Invitrogen) amplified 950 bp of the phylogenetically conserved region of small subunit (SSU) and internal transcribed spacer (ITS) of *Nucleospora cyclopteri* small subunit ribosomal RNA gene (Accession: KC203457). The second specific primer pair (LN2_fwd 5′ctgcttaatttgactcaacgc and LN2_Rev 5′ tactgctcctcaaatagtatg, Invitrogen) amplified 589 bp within LN1 amplicon targeting partially the ITS and SSU regions. For both steps, PCR was carried out in 20 µl reaction volumes containing 10 µl of ready-to-use 2× BioMix™ (Bioline), 15 pmol of each forward and reverse primers, 2 µl of extracted DNA (for 1st reaction) or 1 µl of first PCR-reaction product (for 2nd reaction), adjusted to final volume of 20 µl with autoclaved HPLC grade water (Fisher Scientific). A one-step, conventional PCR was carried out using either the LN1 or LN2 primer pair with 2 µl of extracted DNA in 20 µl reaction for fish that had clinical signs of microsporidiosis. Working concentrations of the initial template for the nested PCR were adjusted to 30 - 60 ng/µl. PCR reactions were carried out on a T100™ Thermal Cycler (Bio-Rad) with 4 mins initialization at 95 °C, followed by 30 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, and 1 cycle for final extension at 72 °C for 7 mins with a hold at 4 °C. No template control and extraction blanks were included in all PCR assays. Pre- and post-PCR analyses were carried out in separate rooms. All DNA extracted samples ($n = 239$) were analyzed with PCR (nested PCR if the first step was negative). All negative samples and 25 randomly chosen positive samples were analyzed in duplicate to estimate accuracy.

Detection of *N. cyclopteri* was assessed by the presence of diagnostic bands of size 950 bp (single-step PCR) or 589 bp (two-step, nested PCR). To confirm specific amplification of the targets, PCR products with expected band sizes were purified using QIAquick PCR Purification Kit (Qiagen) and Sanger sequenced. Sequences were assembled and

Table 1

Results of prevalence and tissue tropism analyses using nested PCR, and conventional PCR methods. Fish 1 to 39 were used for tissue tropism analyses. Highlighted (grey) negative PCR results were confirmed by nested PCR method. Condition indicates presence macroscopic signs of pathology (pathological) or absence (non-pathologic). ‡ represents fish with only kidney sample available, positive1 represents only second round of nested PCR positive, positive2 indicates both round of nested PCR positive, ‡‡ shows Diff Quik® spore stain negative samples, and ‡‡‡ indicates negative by one-step conventional PCR, but positive on second round of nested PCR. "u.t.d" means unable to determine and "n.a" means not available. Fish 30 to 39 were juveniles for which the sex could not be determined.

	Condition	Age (months)	Origin	Sex	Weight (g)	Method	Kidney	Liver	Heart	Spleen	Gills	Gonads	Blood
Fish#1	non-pathological	18	British	male	310	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#2	pathological	18	British	female	278	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#3	non-pathological	18	British	male	170	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#4	pathological	18	British	female	302	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#5	pathological	18	British	male	420	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#6	pathological	18	British	male	230	nested PCR	positive2	positive2	positive2	positive2	positive2	n.a	n.a
Fish#7	pathological	18	British	male	314	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#8	pathological	18	British	male	260	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#9	pathological	18	British	female	596	conventional PCR	positive	positive	positive	positive	positive	positive[‡‡‡]	n.a
Fish#10	pathological	18	British	female	452	conventional PCR	positive	positive	positive	positive	positive	negative	n.a
Fish#11	non-pathological	18	British	male	560	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#12	pathological	18	British	male	474	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#13	pathological	18	British	female	896	conventional PCR	positive	positive[‡‡‡]	positive[‡‡‡]	positive	positive	positive	n.a
Fish#14	pathological	18	British	female	620	conventional PCR	positive	positive	positive[‡‡‡]	positive	positive	positive	n.a
Fish#15	non-pathological	18	British	male	408	nested PCR	positive1	negative	positive1	positive1	positive1	positive1	n.a
Fish#16	pathological	18	Icelandic	female	428	conventional PCR	positive	negative	positive	positive	positive	positive	n.a
Fish#17	pathological	24	British	male	140	conventional PCR	positive	positive	positive	positive	positive[‡‡‡]	positive	n.a
Fish#18	non-pathological	18	British	male	546	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#19	non-pathological	18	British	male	248	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#20	non-pathological	18	British	male	450	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	n.a
Fish#21	non-pathological	18	British	female	550	nested PCR	positive1	positive1	positive1	positive1	positive1	n.a	n.a
Fish#22	pathological	18	British	male	370	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a

(*continued on next page*)

Table 1 (*continued*)

Fish#23	pathological	18	British	male	360	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#24	pathological	18	British	male	380	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#25	pathological	24	British	male	302	nested PCR	positive2	positive2	positive2	positive2	positive2	positive2	n.a
Fish#26	non-pathological	24	British	female	258	nested PCR	positive1	positive1	negative	positive1	positive1	positive1	n.a
Fish#27	pathological	24	British	female	267	nested PCR	positive2	positive2	positive2	positive2	positive2	n.a	positive2
Fish#28	non-pathological	24	British	female	255	nested PCR	positive1	positive1	positive1	positive1	positive1	positive1	positive1
Fish#29	pathological	24	British	male	257	nested PCR	positive2	positive2	positive2	positive2	positive2	n.a	positive2
Fish#30	non-pathological	6	Norwegian	u.t.d	13	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#31	non-pathological	6	Norwegian	u.t.d	13	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#32	non-pathological	6	Norwegian	u.t.d	13	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#33	non-pathological	9	Icelandic	u.t.d	13.3	nested PCR	positive1‡‡	n.a	n.a	n.a	positive	n.a	positive1
Fish#34	non-pathological	9	Icelandic	u.t.d	13.3	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#35	non-pathological	9	Icelandic	u.t.d	13.3	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#36	non-pathological	9	Icelandic	u.t.d	18	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	positive1
Fish#37	non-pathological	9	Icelandic	u.t.d	18	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	n.a
Fish#38	non-pathological	6	Norwegian	u.t.d	18.3	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	n.a
Fish#39	non-pathological	6	Norwegian	u.t.d	18.3	nested PCR	positive1‡‡	n.a	n.a	n.a	positive1	n.a	n.a
Fish#40	non-pathological	24	British	Female	1850	nested PCR	negative	negative	negative	negative	negative	negative	negative
Fish#41	non-pathological	24	Norwegian	Male	264	nested PCR	negative	negative	negative	negative	negative	negative	negative
Fish#42	pathological	24	British	Female	293	nested PCR	negative	negative	negative	negative	negative	n.a	negative
Fish#43	pathological	24	British	Male	510	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive2
Fish#44	pathological	24	British	Male	662	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive2
Fish#45	pathological	24	British	Female	764	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive2
Fish#46	pathological	18	British	Male	140	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive2
Fish#47	non-pathological	24	British	Female	1956	nested PCR	positive1	n.a	n.a	n.a	n.a	n.a	n.a
Fish#48	non-pathological	24	British	Male	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#49	non-pathological	18	British	Male	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#50	pathological	18	British	Male	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#51	pathological	18	British	Male	n.a	conventional PCR	positive	n.a	n.a	n.a	n.a	n.a	n.a
Fish#52	pathological	18	British	Male	n.a	nested PCR	n.a	positive2	n.a	n.a	n.a	n.a	n.a
Fish#53	pathological	18	British	Female	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#54	pathological	18	British	Female	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#55	pathological	18	British	Female	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#56‡	non-pathological	24	Icelandic	Male	244	nested PCR	negative	n.a	n.a	n.a	n.a	n.a	n.a
Fish#57	non-pathological	24	Icelandic	Male	256	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive1
Fish#58	non-pathological	13	Icelandic	Male	n.a	nested PCR	positive2	n.a	n.a	n.a	n.a	n.a	n.a
Fish#59	non-pathological	13	Icelandic	Male	n.a	nested PCR	positive1	n.a	n.a	n.a	n.a	n.a	n.a
Fish#60	pathological	24	British	Male	164	nested PCR	n.a	n.a	n.a	n.a	n.a	n.a	positive2

aligned with Sequencher 4.5.6 software (Gene Codes Corporation) and subjected to a BLAST search for species identification. Both rounds of nested PCR-products from histopathology-confirmed kidney samples ($n = 7$), and randomly selected amplified products from conventional PCR with LN2 primers ($n = 4$) were sequenced.

2.5. Development of a novel real-time PCR (qPCR) assay for *Nucleospora cyclopteri*

Primers were designed using the National Center for Biotechnology Information (NCBI) Primer-Blast using the algorithms of Bustin et al. (2009) and Peirson et al. (2003) to ensure sensitivity and single-amplicon specificity. Predicted secondary structure was minimized using Beacon Designer Software (PREMIER Biosoft) for SYBR® Green chemistry. The SSU region of *Nucleospora cyclopteri* rRNA gene (Accession: KC203457) was used to design the novel P6 primer-pair (P6_fwd 5′ ttgtgaacccagacggg and P6_Rev 5′ atctttaccaaccagagcag, Invitrogen). The target amplicon was 71 bp long with primer GC-content ranging between 45 and 59%. To normalize target gene amplification, a reference gene (*C. lumpus* 12S rRNA) was selected and amplified using the universal primers (12S−V5 Fwd 5′: actgggattagataccc and 12S−V5 Rev. 5′: tagaacaggctcctctag; Riaz et al. (2011).

Target and reference gene amplification efficiencies for comparative C_q were validated using standard curves (tenfold dilution series of control DNA) to ensure that they had similar PCR reaction efficiencies (97.6% and 95.6% respectively). For *N. cyclopteri* positive controls we used both nested PCR and histopathology confirmed kidney samples (n = 7). Stable presence of the 12S ribosomal RNA reference gene in all tested samples was determined by using the Excel-based BestKeeper software tool, where the stability of the reference gene was examined according to the standard deviation of the raw C_q (Pfaffl et al., 2004). Product amplification specificity was confirmed for both the target and reference genes using high-resolution melting curve analysis, gel electrophoresis and by Sanger sequencing of target-gene amplicons (n = 4).

2.6. Determination of relative parasite loads by real-time qPCR quantification

We used the comparative C_q method to estimate relative parasite loads in clinically (n = 12) and non-clinically affected (n = 11) kidney samples. The following amplification protocol was applied to all qPCR reactions for both reference and target genes: 95 °C for 5mins, 40 cycles of denaturation at 95 °C for 0.05mins, annealing/extension at 60.5 °C for 0.30mins, followed by melt-curve analysis from 65 °C to 95 °C (0.05mins each with 0.5 °C increment), where annealing temperature was defined by temperature gradient trial of target gene. All qPCR assays were done in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), using SsoAdvanced™ universal SYBR® Green Supermix (Bio-Rad). The qPCR assay was carried out in a 10 µl reaction containing 5 µl of SYBR® Green Supermix, 0.25 µl of 10 nmol forward and reverse primers, 1 µl of template DNA and 3.5ul of autoclaved HPLC-gradient grade water. For the reference gene, the same reaction volume was used, but the concentration of forward and reverse primers were set at 5 nmol. Non-template controls were added in all reactions.

All experiments were performed with three technical replicates for each biological replicate. The starting DNA template was diluted to 50 ng/µl-100 ng/µl across all tested samples. The relative abundance of the gene of interest was compared between clinically and non-clinically affected kidneys. The reference-gene was amplified in separated wells in the same 96-well-plate. Melt curves, as well as raw fluorescent measurements (C_q values) at which the fluorescent signal was statistically significant above the background, were obtained from default settings of the CFX™ Manager Software, version 3.1 (Bio-Rad) employing a second derivative maximum method.

2.7. Statistical analysis

R 3.6.1 (R Core Team, 2019) was used for all statistical analysis. Prevalence of *N. cyclopteri* was estimated as the proportion of fish that tested positive for microsporidia divided by the total number of fish tested, and 95% binomial confidence intervals were calculated. Binary logistic regression was used to model the presence of *N. cyclopteri* as a function of presence of clinical signs of infection, body mass, stock origin, sex, and age using a generalized liner model with a binomial log-link. Model simplification was assessed by single term deletions using the *drop1* command and the likelihood ratio test. Prevalence in relation to tissue tropism, and sensitivity of a single-step PCR to detect *N. cyclopteri* in different tissues, were assessed by a equality of proportions test. Raw C_q values were filtered based on the presence of pure amplification products using melt-curve analysis prior to the calculation of mean C_q values. Initial concentrations of the target ($R_{0\text{-target}}$) and reference ($R_{0\text{-referene}}$) genes were calculated according to the algorithms given in Peirson et al. (2003) and Liu and Saint (2002). Parasite loads measured by normalized C_q values were compared between kidney samples of fish with and without clinical signs by a Mann-Whitney U test (Goni et al., 2009).

3. Results

3.1. Histopathological analysis

Histopathological analysis of fish with enlarged kidneys (n = 7) revealed severe lymphoid infiltration of the renal interstitium (the intertubular, extraglomerular and extravascular space of the kidney) with many intranuclear and intracytoplasmic eosinophilic structures, consistent with microsporidiosis as reported by Freeman et al. (2013) and Mullins et al. (1994). In severe cases, renal interstitium was replaced with large lymphoblastic cells, suggesting that microsporidiosis was the principal likely cause of morbidity and mortality in these fish. Of the 53 fish examined, 19 fish (36%) had normal, evenly red kidneys with no clinical signs of infection, and 23 (43%) showed various grades of signs of pathology in kidneys, from patchy pallor to uniformly enlarged kidney (renomegaly) with a whitish appearance (Fig. S1). White nodules were also observed in some livers (Fig. S1). Consistent with Freeman et al. (2013), macroscopic pathological changes were mainly observed in the kidneys, and fish with severe renomegaly also displayed other pathological signs such as bilateral exophthalmia, ascites, anaemia and skin lesions. None of the 10 juveniles from Farm 2 displayed any clinical signs of infection. Microscopic examination using Diff Quik® produced negative results in 10 fish with no obvious clinical signs of pathology, while 2.86 µm microsporidial spores were detected in two fish showing pathological signs (Fig. S2). However, this microscopic staining technique mainly targets small spore-stage of microsporidia, approx. 2.53 × 1.04 µm in size for *N. cyclopteri* (Freeman and Kristmundsson (2013), which might not be sufficiently sensitive when the microsporidial load is small (Andree et al., 1998; Garcia, 2002; Ghosh et al., 2014).

3.2. Prevalence assessed by nested-PCR, conventional PCR and macroscopic analyses

Sanger sequencing of positive controls confirmed that both the larger fragment from primary reaction (950 bp), and the smaller fragment from the secondary nested reaction (589 bps) were both from *Nucleospora cyclopteri* (GenBank: KC203457.1), as well as the PCR-products from a one-step (conventional) PCR using the LN2 primer.

For the prevalence analysis, one tissue from each of 43 fish which had also been analyzed macroscopically were obtained depending on accessibility (kidney n = 36, whole blood n = 6 and heart n = 1; Table 1). Fish with positive PCR were considered to be infected with *N. cyclopteri*. None of the blank extractions was positive. When the initial screening of kidney tissues was found to be negative (n = 4), spleen, liver, gills, and heart were also assessed in 3 of the fish to test for false negatives, and these organs were also found to be negative. All PCR products were analyzed by gel-electrophoresis for both rounds of the nested-PCR reactions. The prevalence of *N. cyclopteri* infection assessed by PCR analysis among juvenile and adult farmed lumpfish was 93.3% (95% CI: 84–98%) compared to 51.7% (95% CI: 38–65%) by macroscopic analysis (Fig. 1). This indicates that PCR is twice as sensitive as macroscopic examination for detecting the presence of *N. cyclopteri* and that approximately 50% of lumpfish in our study (26/56) were asymptomatic carriers that did not show any clinical signs of microsporidiosis. Binary logistic regression indicated that only age was a significant predictor of infection status, although the effect was small (LRT = 4.32, df = 1, P = .038; Table 2).

3.3. Tissue tropism

N. cyclopteri was detected with a high prevalence in the 7 tissues we screened, both among individuals with and without pathological signs (Fig. 2a). All negative results from conventional PCR were further analyzed by nested PCR to avoid false negative results (Fig. S3). Results from nested PCR analysis carried out on duplicate random samples

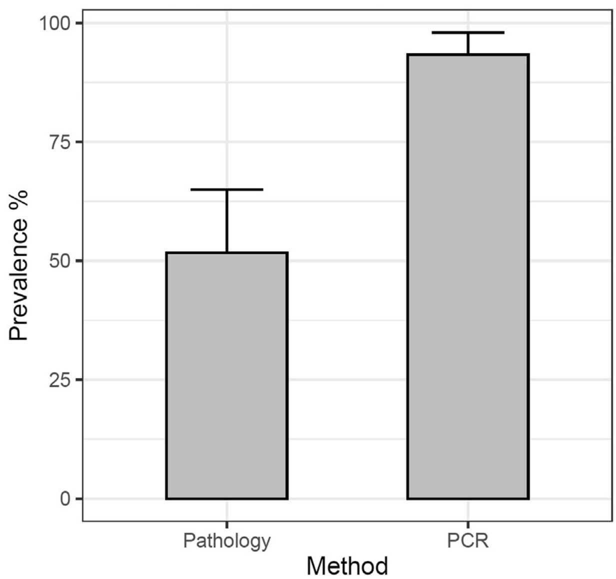

Fig. 1. Prevalence (+ 95% binomial CI) of *Nucleospora cyclopteri* in farmed lumpfish, *Cyclopterus lumpus* (n = 60) investigated by two different diagnostic methods (PCR = 93.3%; Pathology = 51.7%). The more sensitive and specific PCR diagnostic method reveals a much higher prevalence of microsporidial infection among farmed lumpfish.

Table 2
Binary logistic regression of microsporidia infection status in lumpfish detected by PCR, showing results of single term deletions according to the Likelihood Ratio Test (LRT).

Single term deletion	df	Deviance	AIC	LRT	*P*-value
Full model		10.3	26.3		
Pathology	1	10.8	24.8	0.44	0.507
Age	1	14.7	28.7	4.32	0.038
Origin	2	14.9	26.9	4.52	0.104
Sex	2	12.6	24.6	2.28	0.320
Weight	1	11.1	25.1	0.75	0.388

(n = 40) confirmed that there were no false positives. The gills, the kidney and the blood were the tissues that showed the highest prevalence, but no statistical evidence was found for tissue tropism, and all tissues were equally likely to test positive for *N. cyclopteri* (χ^2 = 3.00, df = 6, P = .800). Conventional, single-step PCR detected 48.9% of the samples that were positive with nested PCR, but sensitivity did not differ among tissues (χ^2 = 3.9051, df = 6, P = .686) although the gills, the blood and kidney were again the most sensitive tissues (Fig. 2b).

3.4. Real time PCR analysis

The dissociation curve (the first derivative of the melt curve) for both the reference gene and target gene displayed single specific peaks at T_m values of 83 °C and 82 °C, respectively (Fig. S4). The specificity of the target 71 bp amplicon by the P6 primer pair was further confirmed by Sanger-sequencing (GenBank: KC203457.1). The efficiency of the reference and target amplification reactions was 97.6% and 95.6%, respectively. The limit of detection for the target gene was 1:10^6 dilution of the original pooled DNA (0.0001 ng of input DNA) with a mean C_q value of 34.38 (SD = 0.89).

Mean C_q values from technical replicates were analyzed using the algorithms given in Pfaffl et al. (2004), after discarding poor C_q values (n = 1). Parasite loads were examined in 23 kidney samples of fish that had tested positive with (n = 11) and without (n = 12) pathology signs. Pooled DNA from kidneys that had tested positive for PCR and showed histopathological signs of the disease was used as positive

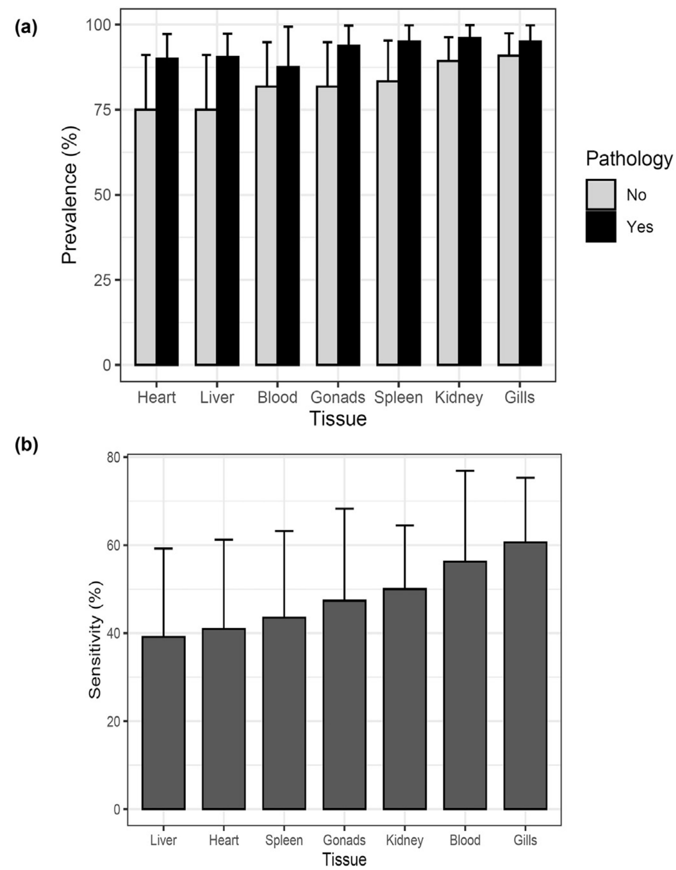

Fig. 2. Variation (+ 95% binomial CI) in (a) prevalence (%) of *Nucleospora cyclopteri* in different tissues of asymptomatic (n = 29) and symptomatic (n = 31) lumpfish using single-step and nested PCR and (b) sensitivity of detection (%) using single-step PCR only.

control. Technical replicates displayed a variation lower than 0.99 with a CV ≤ 2.91% (Table 3), indicating low degree of intra-assay variability and high reproducibility. Normalized C_q median values were much higher for diseased fish (ΔC_q = 0.122), than for asymptomatic carriers ($\Delta C_{q\ =}$ 9.49 × 10^{-7}; Table 3; Mann-Whitney test, χ^2 = 16.5, df = 1, P < .001; Hodges-Lehmann difference = − 0.122), indicating that diseased lumpfish displaying pathological signs of microsporidiosis have a higher parasite load in the kidneys (Fig. 3).

4. Discussion

Our study indicates that *N. cyclopteri* was widespread among the farmed lumpfish we sampled. The proportion of lumpfish with pathological symptoms (52%) was more than twice as high as that reported by Freeman et al. (2013) for wild lumpfish (25%), suggesting that microsporidiosis might be particularly high among farmed stocks. However, many lumpfish that tested positive for *N. cyclopteri* appeared to be asymptomatic carriers (46%), and did not show any clinical signs of microsporidiosis.

The pathological signs of *N. cyclopteri* infection we observed are consistent with previous descriptions of microsporidiosis for lumpfish, and include kidney lymphocytosis and necrosis, as well as evidence of systemic infection (Alarcon et al., 2016; Freeman and Kristmundsson, 2013; Freeman et al., 2013; Mullins et al., 1994). The results of the microscopic and histopathology screening were supported in all cases by the PCR assays.

Our prevalence results (93%) are higher than those reported for *Nosema* microsporidia infection in honeybees (*Apis mellifera;* 63%), but similar to an observed prevalence of 100% for *Pseudoloma neurophilia* in

Table 3
Relative quantification of parasite loads using qPCR. Results for both reference and target genes for kidney samples with and without pathological signs. Normalized C_q values (ΔC_q) were higher for pathological samples in all analyzed specimens indicating higher pathogen concentration. All assays are performed in triplicate at the same time and under the same conditions. SD = standard deviation; CV = coefficient of variation; both SDs and CVs display the variation among technical replicates.

Kidney	Condition	Mean Cq (Reference)	Mean Cq (Target)	Normalized Cq concentration	SDs (reference)	SDs (target)	CVs – reference (%)	CVs – target (%)
1	non-pathology	13.51	34.38	9.49E-07	0.06	0.14	0.415	0.395
2	non-pathology	13.46	34.43	8.84E-07	0.08	0.39	0.612	1.123
3	non-pathology	13.64	33.44	1.95E-06	0.06	0.99	0.460	2.898
4	non-pathology	13.23	34.39	7.78E-07	0.02	0.67	0.137	1.939
5	non-pathology	15.69	33.67	6.72E-06	0.16	0.12	1.027	0.365
6	non-pathology	13.63	33.74	1.58E-06	0.15	0.16	1.100	0.470
7	non-pathology	15.30	35.49	1.53E-06	0.61	0.32	1.340	0.890
8	non-pathology	13.49	34.55	8.33E-07	0.12	0.48	0.909	1.393
9	non-pathology	13.58	35.76	3.93E-07	0.04	0.75	0.328	2.140
10	non-pathology	13.29	34.30	8.59E-07	0.06	0.42	0.441	1.236
11	non-pathology	13.03	31.75	3.99E-06	0.13	0.44	1.026	1.401
12	pathology	14.22	21.15	1.10E-02	0.09	0.05	0.661	0.247
13	pathology	13.68	16.47	1.77E-01	0.35	0.05	2.570	0.300
14	pathology	13.27	16.08	1.73E-01	0.13	0.20	0.952	1.220
15	pathology	14.00	16.96	1.58E-01	0.07	0.18	0.484	1.055
16	pathology	13.40	17.49	7.33E-02	0.04	0.26	0.341	1.466
17	pathology	14.20	17.57	1.20E-01	0.09	0.19	0.636	1.108
18	pathology	16.52	18.50	3.13E-01	0.11	0.04	0.655	0.224
19	pathology	15.52	18.77	1.32E-01	0.09	0.10	0.596	0.518
20	pathology	13.44	26.16	2.23E-04	0.17	0.11	1.268	0.406
21	pathology	13.82	24.45	9.21E-04	0.29	0.50	2.096	2.058
22	pathology	15.57	18.91	1.24E-01	0.12	0.32	0.754	1.712
23	pathology	13.41	17.22	8.85E-02	0.02	0.01	0.174	0.064
24	positive control	15.24	18.75	1.11E-01	0.26	0.10	1.398	0.616

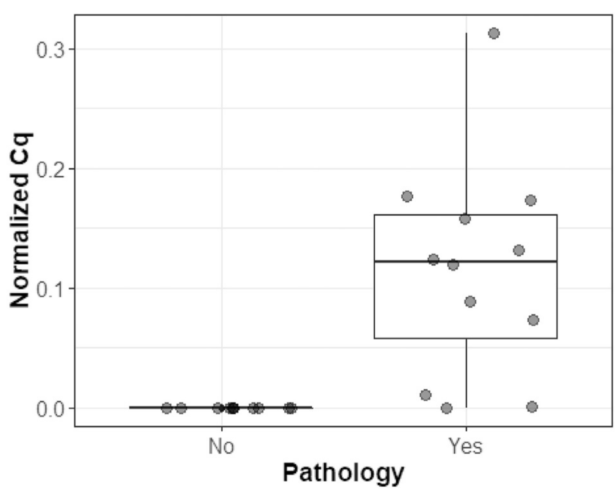

Fig. 3. Distribution of *Nucleospora cyclopteri* loads (normalized C_q concentrations) in the kidneys of asymptomatic (n = 11) and symptomatic (n = 12) lumpfish with microsporidiosis.

experimentally exposed zebrafish (AB strain), and that of *Nucleospora braziliensis* infection in Nile tilapia (*Oreochromis niloticus*) (Papini et al., 2017; Ramsay et al. 2014).

Disease management has become a major concern to meet increasing demand for farmed lumpfish (Alarcon et al., 2016; Powell et al., 2018). Accurate methods of early detection of microsporidia infection, such as those developed here, are urgently needed for epidemiological studies, essential for controlling the outbreak and impact of microsporidiosis in aquaculture (Georgiadis et al., 2001). We found that the nested-PCR assay was more sensitive than microscopic Diff-Quik® staining method, as some stain-negative samples were confirmed to be microsporidia positive by nested PCR. While microsporidia was not detected by one-step conventional PCR in some of the samples from non-pathological fish, it was identified in all nested PCR assays performed in liver, heart, gill, and gonads (Table 1). This confirms that nested PCR is a more sensitive method of detecting microsporidiosis than simple, one-step PCR (Freeman et al., 2013), which might give false negatives on asymptomatic fish having very low parasite loads, as previously observed in chinook salmon (Andree et al., 1998).

The results of the tissue tropism analyses revealed that once lumpfish are infected, microsporidia can spread quickly to almost all the host tissues, probably via peripheral blood dissemination. This type of systemic infection has also been observed in other microsporidians of the genus *Nucleospora*, including *N. braziliensis* infecting Nile tilapia and *Nucleospora salmonis* infecting chinook salmon, both closely related to *N. cyclopteri* (Andree et al., 1998; Rodriguesm et al., 2014). The presence of *N. cyclopteri* in the gonads of both male and female lumpfish suggests that this pathogen could be transmitted vertically from infected parents to offspring (Freeman et al., 2013; Dunn et al., 2001), as for other microsporidia in fish (Sanders et al., 2013), although more work is needed for this to be confirmed. Detection of pathogen from blood (parasitemia) suggested that the systemic infection of *N. cyclopteri* could be accomplished via vascular migration, and that peripheral blood might facilitate the widespread presence of pathogens in all examined tissues.

The PCR detection of *N. cyclopteri* in gills and blood offers a rapid, non-lethal screening of microsporidia with great potential for use in aquaculture. Kidney and gills are expected to be the primary sites of infection, as well as the main routes of transmission for microsporidian infections (Freeman et al., 2013), but very little is known about the epidemiology of the disease. In this sense, our novel qPCR assay should be particularly useful as it can be used to quantify parasite loads across different tissues and examine the routes and mechanisms of transmission in more detail.

Currently, spore staining and histopathology are the diagnostic tests most commonly used to evaluate the degree of microsporidian infection, but they lack sensitivity and specificity, and are time consuming. The newly developed real-time qPCR assay is not only more sensitive (can detect infection in fish with no clinical signs), faster and specific, it can also estimate relative parasite loads, providing quantitative measures of infection. The qPCR assay is also faster than nested PCR, and involves fewer steps which lessens the risk of cross-contamination between samples.

Lumpfish showing clinical signs of microporidiosis displayed a much higher parasite load than asymptomatic carriers, suggesting that the morbidity of the disease may follow a dose-dependent response, as shown for other microsporidia (Didier et al., 2006). It is possible that the chronic and sub-clinical forms of *N. cyclopteri* infection are the norm, as seen in *N. salmonis* infecting salmonids, and that individuals with low parasite loads act as reservoirs for *N. cyclopteri* in lumpfish, which may explain its very high prevalence (Hedrick et al., 2012). Latent infections have also been observed for other mirosporidians, such as *E. cuniculi* detected in immunocompetent humans and *Desmozoon lepeoptherii* infecting Atlantic salmon (Didier and Weiss, 2011; Didier et al., 2004; Freeman and Sommerville, 2011; Ramsay et al., 2009; Sestak et al., 2003). Our study suggests that *N. cyclopteri* could also be latent in healthy lumpfish, which could progress to disease with the proliferation of the parasite and tissue damage during the immunocompromised stages. Thus, it is possible that differences in parasite loads and in pathogen susceptibility among individuals might have a genetic basis, as it has been observed in the Atlantic cod infected by the microsporidian *Loma morhua* (Frenette et al., 2020), and that close proximity or stress caused by aquaculture conditions might contribute to the spread of the disease (de Hoog et al., 2011; Sitja-Bobadilla et al., 2016). It is also possible that asymptomatic lumpfish with low parasite loads might indicate early stages of *N. cyclopteri* infection.

In summary, our study represents the first PCR-based analysis of tissue tropism of *N. cyclopteri* and the first quantitative comparison between pathological and asymptomatic lumpfish. The results indicate that asymptomatic lumpfish can have a very high prevalence of *N. cyclopteri,* and that microsporiodosis in lumpfish is consistent with systemic infection via the peripheral blood, despite lack of pathological signs in some fish. Infection status was not influenced by sex, body size, or stock origin, despite large genetic differences between stocks (Whittaker et al., 2018). Many infected fish appeared to be macroscopically healthy, suggesting that the manifestation of pathological signs may depend on the stage of the disease as well as the host immune condition. Both the nested PCR and qPCR assays proved more sensitive than traditional methods for diagnosing sub-clinical infections, but our novel qPCR assay offers several additional advantages, including a higher throughput, a faster turnaround and, crucially, a quantitative assessment of microsporidian loads.

Ethical approval

Ethical approval for this research was granted by Swansea University (Biosciences Ethics Committee, BS26/01/2017). The authors have no competing interests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735779.

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