Title: Detection of the Intranuclear Microsporidian *Enterospora nucleophila* in Gilthead Sea Bream by *In situ* Hybridization

Short running title: ISH detection of *E. nucleophila*

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ABSTRACT

Enterospora nucleophila is an intranuclear microsporidian responsible for emaciative microsporidiosis of gilthead sea bream (GSB). Its minute size and cryptic nature make it easily misdiagnosed. An *In situ* hybridization (ISH) technique based on antisense oligonucleotide probes specific for the parasite was developed and used in clinically infected GSB in combination with calcofluor white stain (CW) and other histopathological techniques. The ISH method was found to label very conspicuously the cells containing parasite stages, with the signal concentrating in merogonial and sporogonial plasmodia within the infected cell nuclei. Comparison with CW demonstrated limited ISH signal in cells containing mature spores, that was attributed mostly to the scarcity of probe targets present in these stages. Although spores were detected in other organs of the digestive system as well as in the peripheral blood, proliferative stages or parasite reservoirs were not found in this work outside the intestines. The study demonstrated a frequent disassociation between the presence of abundant spores and the intensity of the infections as determined by the parasite activity. The ISH allows confirmatory diagnosis of GSB microsporidiosis and estimation of infection intensity, and will be a valuable tool for a more precise determination of parasite dissemination pathways and pathogeny mechanisms.

Keywords: Parasite, Microsporidia, Enterospora, In-situ Hybridization, Diagnostics

1 INTRODUCTION

2 Microsporidians are minute intracellular parasites that infect all animal phyla and even
3 protists like amoebae and gregarines (Larsson 2000, Scheid et al. 2008). Over half of
4 the known genera infect aquatic organisms (Stentiford et al. 2016). With a close
5 phylogenetic relationship with fungi, they share biological features such as the
6 presence of chitin in the spores, the use of trehalose as the main sugar reserve or
7 some similarities during mitosis and meiosis (Han and Weiss, 2017). Like in the
8 Rozellomycota, adaption to obligate intracellular parasitism has led to gene loss,
9 genome reduction and dependence upon the host for cell function (Quandt et al, 2017,
10 Ndikumana et al., 2017). While many species tend to be considered secondary
11 pathogens or linked to immunocompromised condition in human or veterinary
12 medicine, numerous microsporidioses are involved in serious losses in aquaculture
13 settings (El Alaoui et al, 2006, Kent et al. 2014, Palenzuela et al. 2014, Stentiford et al.
14 2016).
15 Multiple species of microsporidians have been reported in gilthead sea bream, *Sparus*
16 *aurata* (GSB) (Faye et al. 1988, Abela et al. 1996, Athanassopoulou 1998, Morsy et al.
17 2013, Mathieu-Daude et al.) but seldom related to clinical diseases in aquaculture
18 settings. An intranuclear species was found in Spanish GSB farms since the 00s, and its
19 association with emaciative disease and dropping mortality was further established as
20 new cases appeared in different facilities. This parasite was described as *Enterospora*
21 *nucleophila* and its closest known relative is *Enterocytozoon hepatopenaei* (Palenzuela
22 et al. 2014), a serious shrimp pathogen widespread in Asia (Thitamadee et al. 2016,
23 Rajendran et al. 2016) and, recently, in South America (Tang et al. 2018). Since the
24 emergency of the disease, it has been also detected in Italian and Greek GSB farms,

and in different facilities including on-growing net cages and land-based GSB nurseries (Caffara et al. 2014 and authors' unpublished data). However, clinical infections only affect certain fish lots or cages and they are not usually widespread within a given facility. In addition, clinical presentation does not always correlate with detection of microsporidian spores in large quantities and, likewise, presence of the parasite has been registered in the absence of clinical symptoms. While specific PCR tests are an invaluable tool for parasite surveillance and epidemiology, interpretation of the disease dynamics and risk assessment are difficult to approach without deep knowledge of the parasite development and pathogenesis.

The diagnosis of *E. nucleophila* is quite challenging due to its tiny size (spore width is about 1 µm) and relatively sparse intranuclear development in certain intestinal epithelial cells. The detection in histology slides is mainly limited to the spore stage, and even these are difficult to identify in routine slides except when present in large quantities. Use of fluorescent brighteners binding to chitin (e.g. calcofluor white) for the detection of microsporidians largely improves the sensitivity in these contexts (Freeman et al. 2013, Palenzuela et al. 2014, Alarcón et al. 2016, Herrero et al. 2018), yet it only facilitates the detection of spores which is the only stage stained. In situ hybridization techniques have been successfully used to circumvent similar limitations in the closely related microsporidian *Enterocytozoon hepatopenaei* (Tang et al. 2015, Rajendran et al. 2016), or in *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) (Weli et al. 2017). The main objective of this work was to develop an ISHbased technique to detect *E. nucleophila* stages, getting insights into the development of the parasite and the pathogenesis of GSM emaciative microsporidiosis.

MATERIALS AND METHODS

Fish

Sparus aurata specimens were collected from a Spanish GSB offshore net pen farm located at Southern Castellón (Comunidad Valenciana region). The facility had a background of *E. nucleophila* infections detected in previous surveys by our laboratory. During the winter season of 2016, a cage showing clear growth retardation and anorexia, abnormally high mortality rate and size segregation within the stock, which are signs of presumptive *E. nucleophila* infection in GSB (Palenzuela et al., 2004), was sampled. Fish were harvested using nets and the smaller, wasted fish were handpicked by the pathologists. Approximately 250 specimens (average weight: 43.4 g) were transferred alive to the Institute of Aquaculture “Torre de La Sal” (IATS) and maintained in 500L fiberglass tanks supplied with a flow-through natural water supply. The specimens were anaesthetized, sacrificed and sampled following routine procedures in compliance with European (86/609/EEC) and National (Royal Decree RD1201/2005) for the protection of animals used in scientific experiments, and approved by the CSIC ethics committee and IATS Review Board. A total of 60 individual fish were euthanized and sampled for downstream analyses. During the necropsies, small pieces of the anterior, middle and posterior intestine were dissected. Samples from different organs were also collected (liver, spleen, stomach, skeletal muscle, gall bladder, brain, gonads, head kidney and trunk kidney). Tissue pieces were immediately transferred to histology cassettes and fixed in 10% neutral buffered formalin for 1-2 weeks, before embedding into paraffin blocks following routine histological procedures. Some subsamples of tissue were embedded into methacrylate resin Technovit 7100 (Kultzer, Werheim, Germany).

Histology and calcofluor white staining.

Paraffin sections (5 µm thickness) corresponding to the organs of 60 fish were cut and mounted onto electrostatically charged glass slides (Superfrost-Plus, Thermo Fisher Scientific, Spain). They were stained with 0.1% calcofluor white M2R stain (CW) in ddH2O with a drop of 10% potassium hydroxide, for 1 min. The slides were rinsed immediately with 30% ethanol. A light counterstaining was achieved with 0.1% Evans blue solution containing a few drops of glacial acetic acid for 1 min. Slides were examined using an Olympus BX51 fluorescence microscope under UV excitation light. Plastic sections (1 µm) were routinely stained with toluidine blue and examined under visible light.

E. nucleophila probes design and In Situ Hybridization (ISH) procedure

The small subunit ribosomal DNA sequences of *E. nucleophila* isolates (Palenzuela et al. 2014) were used as target. A dataset containing all the sequences available under the category “microsporidia” (2,450 entries, 209 Enterocytozoonidae) in the SSU_r132 database release by SILVA (Pruesse et al. 2007, www.arb-silva.de) was downloaded. The alignment was pruned to the closest related Enterocytozoonidae sequences and then refined manually according to secondary structure criteria using ARB software (Ludwig 2004). Unique *E. nucleophila* oligonucleotide segments, or segments with sufficient variation with respect to the closest taxa, were visually identified in the alignment. Oligonucleotide antisense probes were designed to hybridize with the positive strand of the gene (i.e., binding to both rDNA and rRNA of the parasite) at these regions. Choice of the best probes according to specificity and thermodynamic properties was aided by the software package OLIGO-7 (Rychlik 2007). Two

97 oligonucleotides were chosen: Enu_725L25 (5'TCC CAC ACC AA CAC CAC TTT CAT A-3')
98 and Enu_101L25 (5'-ATC CGT TCC GCC ATC TAT GTA CAT C-3'). They were modified
99 with 5' digoxigenin labels and sourced from a commercial supplier (Metabion Int.,
100 Germany), suspended at 100 uM in TE, and mixed in equimolar amounts as a
101 concentrated stock.
102 A panel of 32 samples was chosen representing individuals with assorted infection
103 status as determined by the results of the histopathological examination, including
104 Individuals in which few or no microsporidian spores were detected with CW. The ISH
105 procedure was carried out with these probes as described previously for other fish
106 parasites (Palenzuela & Bartholomew 2002, Cuadrado et al. 2007, Constenla et al.
107 2016), with some modifications. Deparaffinized and hydrated sections were treated
108 with 15 $\mu g.ml^{-1}$ proteinase K (45 minutes at 37 ^{o}C). They were then washed, denatured,
109 and incubated overnight at 37 ^{o}C with the mixture of probes diluted 1:500 in
110 hybridization buffer. Stringency washes were made twice in each decreasing
111 concentrations of SSC buffer (2x, 1x, and 0.25x), for 20 min each, at 40 ^{o}C under a
112 gentle rocking motion. The slides were equilibrated in 1x Genius Buffer at RT and the
113 immunological detection was conducted with AP-conjugated Fab fragments from
114 AntiDig sheep antiserum (Roche Diagnostics, Manheim, Germany), diluted 1:750 in
115 blocking solution and incubated for 2 hours at RT. The colorimetric reaction was
116 performed with NBT/BCIP substrate (nitro-blue tetrazolium chloride/ 5-bromo 4-
117 chloro- 3'indoly phosphate- p toluidine salt) for 2-8 hours at room temperature, until
118 optimum signal with low background was achieved. The slides were counterstained
119 with light green (1%), mounted in permanent medium and observed by light
120 microscopy.

RESULTS AND DISCUSSION

The histopathological study of clinically infected samples stained with fluorescent brightener calcofluor white M2R clearly showed the localization of *E. nucleophila* spores within the nuclei of enterocytes and rodlet cells (RCs), typically bunched in a berrylike pattern clustering up to 25 spores (Fig. 1A). Spores were also detected in the connective tissue and submucosae, either scattered or in large clusters, the later most often within or around melanomacrophage centers (MMCs) (Fig. 1B). The findings were consistent with previous histopathological and ultrastructural studies of this infection (Palenzuela et al. 2014) demonstrating the primary intranuclear development of the parasite in epithelial cells and its presence in cytoplasmic position within other cells, including phagocytes at subepithelial layers. However, the increased sensitivity of the fluorescent stain facilitated the detection of scattered spores in other locations, as a consequence of degenerative changes in the epithelium, spore detachment, and host cellular immune responses (Palenzuela et al. 2014, Sitja-Bobadilla et al. 2016). Scarce spores were found in stomach, gall bladder, peripheral blood, or liver samples (Figs. 1C, 1D, and data not shown). Calcofluor white M2R is widely used to detect microsporidian species due to the high affinity of this compound to bind the chitin present at the microsporidia endospore membrane (Han and Weiss, 2017). Other chemofluorescent agents, mostly stilbene derivatives typically used as whiteners in detergents and in paper and fabrics manufacture, present similar properties acting as chitin-specific stains (e.g., Hoch et al 2005, Rüchel & Schaffrinski 1999). Their use is considered a credible technique for the diagnosis of microsporidians (Didier et al 1995, García, 2002). However, these stains only bind to spores and not to other stages of

145 microsporidian cells devoid of chitin, and therefore other strategies are needed to
146 study microsporidian cells development in the host (Chen et al, 2017).
147 The ISH procedure produced intense blue-purple signals in clinically infected fish,
148 particularly in the intestinal mucosa (Fig. 1E). Detailed examination and double staining
149 with ISH and CW showed that the bulk of the ISH signal concentrated in cells
150 containing microsporidians under proliferative, pre-sporogony development phase and
151 in which *E. nucleophila* spores were not present (Fig. 1F). Indeed, scarce ISH signal was
152 present in mature spores which appeared mostly unstained when present (Fig.1G).
153 This pattern resulted quite revealing for the confirmation of the infection in samples
154 presenting the typical epithelial hypercellularity and nuclei alterations (Fig. 1H) often
155 associated to the *E. nucleophila* infection (Palenzuela et al. 2014), but in which
156 confirmatory diagnosis was not possible due to the absence of spores in routine
157 histopathological examination or in calcofluor-stained sections. The staining was a
158 clear result of the design of antisense probes targeting parasite rRNA present in the
159 infected host cells, most often within the nuclei (Fig 1I). This approach resulted in
160 enhanced sensitivity and, more importantly, in the ability to detect metabolically
161 active parasite stages containing many ribosomes. Light but noticeable ISH background
162 was present in some samples from infected stocks in which neither spores nor clearly
163 ISH-positive cells were present, but presenting large numbers of granulocytes and
164 other histopathological alterations associated to the infection (not shown). Since
165 background signal was never present in control uninfected samples or in control slides
166 without probes, it was interpreted to be caused by parasite remnants present in late
167 infections. However, it was not possible to determine if these patterns indicated a

recovery stage or a covert infection from which the parasite development could be reactivated.

The scarce or absent ISH signal in mature spores is most likely related to a low number of probe targets. Since scarce or null protein synthesis activity is present in mature spores, the signal is mostly limited to parasite genomic rDNA copies. Some microsporidians have extremely condensed genomes and scarce ribosomal gene arrays, like *Encephalitozoon intestinalis* with the smallest known nuclear genome (2.3Mbp) and only 11 rDNA arrays per haploid genome (Corradi et al. 2010). Enterocytozoonidae species closely related to *E. nucleophila* are included in this trend, with recent genome assemblies of 3.1 and 3.26 Mbp in *Enterospora canceri* and *Enterocytozoon hepatopenaei*, respectively (Wiredu Boakye et al. 2017). However, the low signal intensity observed in spores could also be partly related to incomplete permeabilization of their shells. A very similar ISH staining pattern was described with anti-sense RNA probes for the salmon microsporidian *Desmozoon lepeophtherii*, which labelled strongly ribosome-rich merogonial and plasmodial stages (Weli et al. 2017). In our procedure, the use of pre-labelled synthetic oligonucleotide probes instead of longer lab-made RNA probes and has obvious advantages for practical purposes. Even though ISH allowed clear detection of *E. nucleophila* developmental stages in GSB tissues, these were not found in other organs except the intestine. Using CW staining, however, microsporidian spores were occasionally found isolated or in small clusters in peripheral blood, skeletal muscle, gall bladder, liver and stomach in some clinically infected fish. These appear to be related to systemic spreading of advanced infections and parasite clearing mechanisms more than to previously unidentified parasite development niches in the fish. This observation is consistent with the pathogeny of

192 the disease, which is mostly evidenced as a chronic severe emaciation and growth
193 arrestment due to intestinal dysfunction. By ISH, it was evidenced that heavy
194 infections in clinically infected fish are not consistently associated with the presence of
195 large numbers of spores and, likewise, these tend to be present more abundantly in
196 samples with limited overall parasite activity. The limited epidemiological data on
197 emaciative microsporidiosis of GSB points to it as a chronic condition, as it results in
198 size segmentation of infected and uninfected fish within the same cage, reaching levels
199 equivalent to year-class differences in some cases (Palenzuela et al. 2014 and author's
200 unpublished observations). This suggests either a very slow development cycle or an
201 equilibrium between clearing of spores and reinfection of regenerated epithelia. The
202 development in the intestinal mucosa certainly opens the path for a direct release and
203 dispersion with fecal matter as in shrimp *E. hepatopenaei* (Tang et al. 2016). However,
204 extensive detachment of the epithelium and sloughing of tissue ribbons, like in the
205 GSB-infecting myxozoan *Enteromyxum leei* (Sitja-Bobadilla & Palenzuela 2011) are not
206 commonly observed in this infection. More likely, the results point to a late and
207 relatively modest sporulation prolificacy within the fish. Clearance of spores by
208 phagocytes and accumulation in MMC was observed, but it was mostly found in
209 advanced infections with little parasite load and activity. Although *E. nucleophila* is
210 rooted in a clade of crustacean-infecting enterocytozoonids (Palenzuela et al. 2014) in
211 which copious sporulation is the norm, the existence of alternate hosts for this species
212 is yet unknown.
213 In summary, the ISH procedure developed in this work appears as a valuable tool for
214 the confirmatory diagnosis of *E. nucleophila* infections in GSB. Although calcofluor
215 staining is an easy method to detect spores, their presence and amount was often

found to be disassociated of the true infection intensity in terms of number of infected host cells. Using the ISH in combination with calcofluor fluorescence staining and conventional histopathological techniques in a stock of clinically infected GSB, we found that the parasite restricts its proliferation and development to the intestine epithelium although isolated spores can reach peripheral blood, and occasionally other digestive and blood-rich organs. Determination of more precise parasite dissemination pathways and pathogeny mechanisms depend on the ability to conduct experimental infections and time-course infection analyses with this parasite.

CONFLICT OF INTEREST STATEMENT

The Authors declare that there is no conflict of interest.

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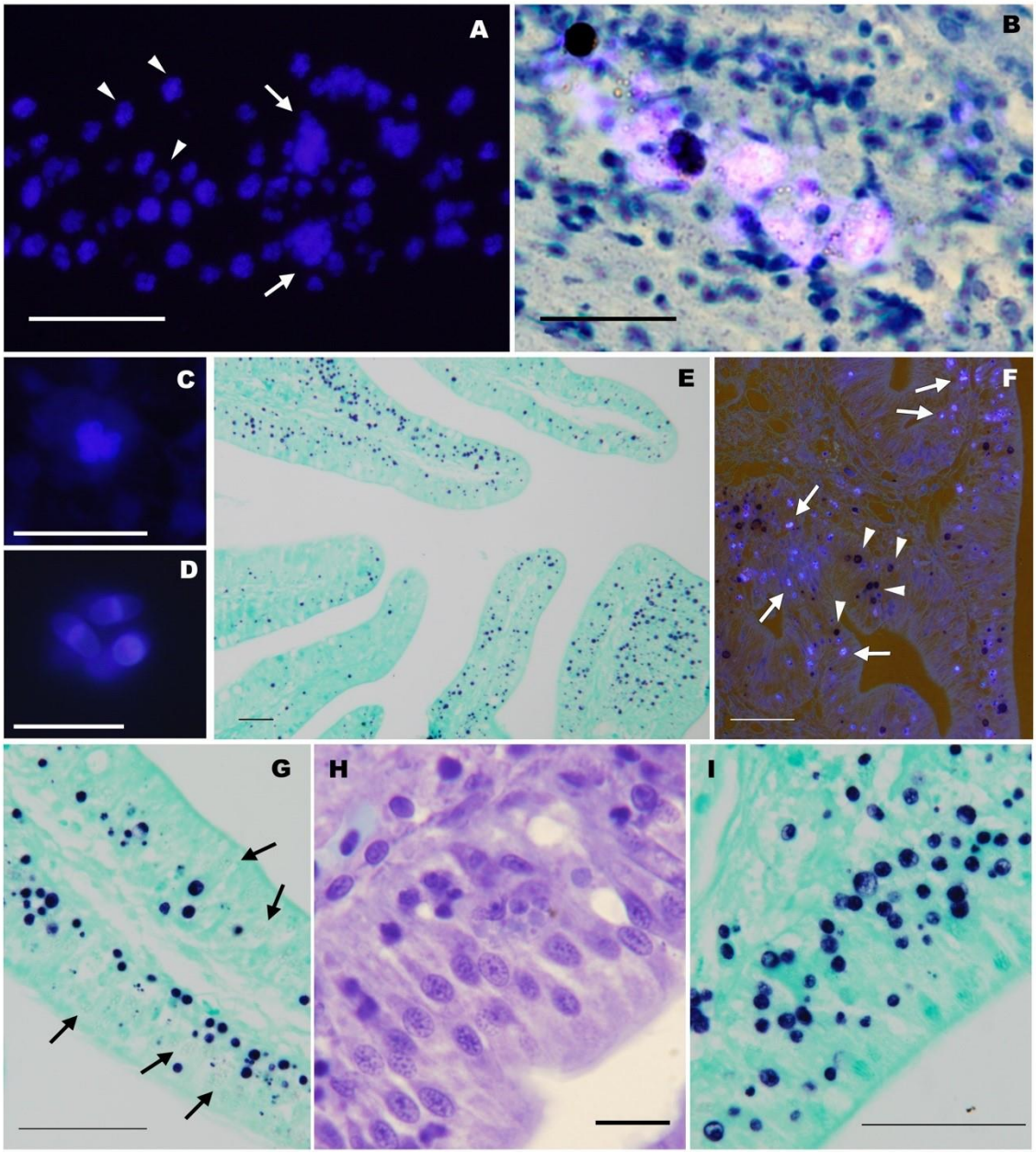
FIGURE LEGENDS

Figure 1: Histology sections of gilthead sea bream tissues infected by *Enterospora nucleophila*. A: Infected intestine epithelium showing clusters of intranuclear (arrowheads) and cytoplasmatic (arrows) spores (calcofluor white stain, CW). B: Detail of a melanomacrophage center (MMC) at the intestinal submucosa. Note the bright calcofluor-positive material accumulated within the MMC and in some microsporidian spores at the periphery (double stain with toluidine blue and CW). C & D: *Enterospora nucleophila* spores in the gall bladder and the blood, respectively (CW). E: Panoramic view of a heavily infected intestine stained with the ISH procedure, showing conspicuous purple positive cells contrasting with the light green counterstain. F: Panoramic view of an infected intestine. Note the different staining patterns with the ISH (strongly positive cells containing replicating stages stained as deep purple, arrowheads) and the CW (bright blue clusters of mature spores, arrows) (double stain with CW and ISH, photographed under simultaneous visible and UV light). G: Detail of an intestinal epithelium containing –different developmental stages of *E. nucleophila*. Mature spores are mostly unstained (arrows). H: Toluidine blue-stained plastic section showing the typical hypercellularity and altered nuclei often associated with *E. nucleophila* infection. I: Detail of ISH-staining pattern in a similar infection to that shown in H. Note the ISH signal with variable intensity in different cells, proportional to the parasite activity and concentrating within the cell nuclei. Scale bars: 20 µm (A & B); 10 µm (C); 5 µm (D); 50 µm (Figs. E, G & I); 25 µm (F); 10 µm (H).



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