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Malacosporean myxozoans exploit a diversity of fish hosts

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Abstract

Myxozoans are widespread and common endoparasites of fish with complex life cycles, infecting vertebrate and invertebrate hosts. There are two classes: Myxosporea and Malacosporea. To date some 2,500 myxosporean species have been described. By comparison, there are only five described malacosporean species. Malacosporean development in the invertebrate hosts (freshwater bryozoans) has been relatively well studied but is poorly known in fish hosts. Our aim was to investigate the presence and development of malacosporeans infecting a diversity of fish from Brazil, Europe and the USA. We examined kidney from 256 fish belonging variously to the Salmonidae, Cyprinidae, Nemacheilidae, Esocidae, Percidae, Polyodontidae, Serrasalmidae, Cichlidae and Pimelodidae. Malacosporean infections were detected and identified by PCR and SSU rDNA sequencing, and the presence of sporogonic stages was evaluated by ultrastructural examination. We found five malacosporean infections in populations of seven European fish species (brown trout, rainbow trout, white fish, dace, roach, gudgeon and stone loach). Ultrastructural analyses revealed sporogonic stages in kidney tubules of three fish species (brown trout, roach and stone loach), providing evidence that fish belonging to at least three families are true hosts. These results expand the range of fish hosts exploited by malacosporeans to complete their life cycle.

Key words: *Buddenbrockia*, *Tetracapsuloides,* SSU rDNA sequence, ultrastructure, sporogenesis, Myxozoa, Cnidaria.

Key findings

- Infections of five malacosporean species were detected in populations of seven European fish species.
- Sporogonic stages were observed in kidney tubules of three fish species.
- Molecular and ultrastructural data provided evidence that fish belonging to at least three families are true hosts.
- A second malacosporean life cycle is resolved.

Introduction

Myxozoans are microscopic, obligate, endoparasitic cnidarians with complex life cycles (Okamura *et al.,* 2015a). Transmission from host to host is achieved by multicellular spores whose morphologies have been used extensively for taxonomic purposes. However, as it became clear that convergence in spore morphotypes could be problematic, researchers have increasingly incorporated small subunit ribosomal DNA (SSU rDNA) sequences as additional data for the reliable identification of species (Kent *et al.,* 2001; Atkinson *et al*., 2015). Myxozoans are comprised of two lineages: the speciose Myxosporea and the species-poor Malacosporea. Collectively there are some 2,500 described myxozoan species (Okamura *et al.,* 2018). Myxosporean lifecycles involve annelids as definitive hosts and vertebrates, mainly fishes, as intermediate hosts. Malacosporeans use freshwater bryozoans as definitive hosts and fish as intermediate hosts.

To date only five malacosporean species have been described (Patra *et al.,* 2016). There are two malacosporean genera: *Tetracapsuloides* and *Buddenbrockia.* Species in both genera develop as sac-like or vermiform (myxoworm) stages in the body cavity of their freshwater bryozoan hosts (Hartikainen et al. 2014). Spores produced within sacs and myxoworms are infectious to fish. The only malacosporean whose life cycle has been resolved and whose development in both hosts has

been characterized is *Tetracapsuloides bryosalmonae*, the causative agent of salmonid proliferative kidney disease (PKD). *T. bryosalmonae* develops as sacs in the body cavity of freshwater bryozoans (Anderson *et al*., 1999; Canning *et al.,* 1999) and as pseudoplasmodia in kidney tubules of salmonid fish (Kent and Hedrick, 1985). Spores released from bryozoans infect fish (Feist *et al.,* 2001) and spores passed with fish urine infect bryozoans (Hedrick *et al.,* 2004; Morris and Adams, 2006).

Recent studies provide evidence of a further twelve undescribed species of malacosporeans from a diversity of bryozoan and fish hosts (Bartošová-Sojková *et al.,* 2014; Hartikainen *et al.,* 2014; Patra *et al.,* 2016). These results suggest that there is substantially greater diversity of malacosporeans than is currently appreciated and that further investigations may link malacosporeans detected in fish with those detected in bryozoans, thereby resolving life cycles. The detection of undescribed malacosporeans in fish has, however, largely been gained by PCR and sequencing of material from fish kidney without ascertaining spore development in the putative fish hosts. It is therefore possible that putative fish hosts may be accidental (Bartošová-Sojková *et al.,* 2014; Hartikainen *et al.,* 2014). For example, larvae of some nematode parasites (e.g. *Ancylostoma braziliense, Ancylostoma caninum, Toxocara canis* and *Gnasthostoma spinigerum*) may begin their development in humans but only develop to mature adult worms in their true mammal hosts (Rey, 2008). Similarly, malacosporeans could invade fish as blood stages that are detected by PCR of fish kidney but fish may not support subsequent spore development. Indeed, this has been observed when local *T. bryosalmonae* strains infect exotic rainbow trout in the United Kingdom (Bucke *et al.,* 1991; Morris *et al.,* 1997).

The aim of this study was to characterize malacosporeans in a diversity of fish kidney material, employing both molecular and ultrastructural methods. By using this combined approach, we are able to confirm that malacosporeans exploit fish hosts belonging to at least three families. In addition, we are able to resolve a second malacosporean life cycle by linking the vertebrate and

invertebrate hosts. Extending our knowledge of malacosporean host diversity is of general importance for understanding biodiversity, ecology and co-evolutionary relationships in freshwater systems and could be relevant for diagnosis and control of emerging diseases in aquaculture or wild fish populations in our changing world.

Material and methods

A total of 256 fish kidney were screened for the presence of malacosporean DNA. The material we studied was gained by a mixture of general and targeted sampling. The former involved taking advantage of ongoing project work sampling fish in the River Stour (electrofishing) and in Blickling Lake (rod fishing), and screening for fish parasites in practical classes in Switzerland (caught by net). More targeted sampling included material collected during surveys for parasites in paddlefish in the USA and by specifically sampling fish on farms in Brazil. Nineteen fish species belonging to 9 fish families (Salmonidae, Cyprinidae, Nemacheilidae, Esocidae, Percidae, Polyodontidae, Serrasalmidae, Cichlidae and Pimelodidae) were sampled from the United Kingdom (UK), Switzerland, Brazil and the United States of America (USA) (Tables 1 and 2). We included archived samples of brown and rainbow trout that were known to be infected by *T. bryosalmonae* to provide comparative material because the development of *T. bryosalmonae* is well known. The fish were euthanized in Brazil by benzocaine overdose, in accordance with Brazilian law (Federal Law No. 11.794, dated 8 October 2008 and Federal Decree No. 6899, dated 15 July 2009), and in Europe by a blow to the head, followed by severance of the spinal cord. Approximately 27mm^3 of tissue was immediately dissected from the posterior portion of the kidney. One half of the kidney material was fixed in 99% ethanol for the molecular analysis and the other half in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer for the ultrastructural studies described below.

DNA extraction, PCR amplification, sequencing and species identification

The DNA was extracted using a DNeasy® Blood & Tissue kit (Qiagen, USA), following the manufacturer's instructions. Malacosporean specific mala-f and mala-r primers (Grabner and El-Matbouli, 2010) were used in PCRs for all samples, amplifying approximately 680 bp of the SSU rDNA. Malacosporean-specific budd-f and budd-r primers (Grabner and El-Matbouli, 2010) were then subsequently used to amplify almost complete length SSU rDNA giving a product that is approximately 1784 bp. General myxozoan primers such as MedlinA and MedlinB (Medlin *et al.*, 1988) were trialed in pilot work but did not amplify any malacosporeans.

Polymerase chain reactions (PCRs) were carried out in 25 µL reaction volumes using 100 ng of extracted DNA, 5 ×Go Taq Flexi Buffer (Promega Madison, WI, USA), 10 mM dNTP mix, 25mM MgCl2, 10mM for each primer and 1×GoTaq G2 Flexi DNA polymerase (Promega, Madison,WI, USA). The original cycling conditions were used for mala-f and mala-r primers as described by Grabner and El-Matbouli (2010). For runs using budd-f and budd-r primers an initial denaturation stage at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 61 °C for 45 s, extension at 72 °C for 105 s, finishing with an extended elongation stage at 72 °C for 8 min. The cycling conditions were modified from Grabner and El-Matbouli (2010) for budd-f and budd-r primers to increase primer specificity.

Ultrastructural investigation suggested the presence of sphaerosporid myxozoans in kidney of white fish and dace. To identify cases presenting simultaneous infections of both sphaerosporids and malacosporeans all kidney material was screened by nested PCR using the primers and conditions outlined in Patra *et al.*, 2018 (Erib 1 and 10 primers for primary PCR, [95 °C for 5 min, 35 cycles of 94 °C for 1 min, 60°C for 1 min, extension 90 s] followed by final extension of 5 mins and SphFWSSU1243F and SphFWSSU3418R for nested PCR primers [95 °C for 5 min, 35 cycles of 94 °C for 1 min, 56°C for 1 min, extension 90 s] followed by final extension of 5 mins).

PCR products were electrophoresed in 2.0% agarose gel, stained with gel red and analysed by a Syngene Transilluminator. PCR products were purified using a Gel/PCR DNA Fragment

Extraction Kit (Geneaid Biotech Ltd., USA) and sequenced. This work was conducted in the Molecular Biology Unit of the Natural History Museum, London (NHM) using the Applied Biosystems 3730xl DNA Analyser for Sanger sequencing. OTUs were compared with SSU rDNA sequence data in GenBank and species identity was based on ≥99% similarity (Bartošová-Sojková *et al*., 2014; Bartošová and Fiala, 2011; Whipps and Kent, 2006). An alignment of the original SSU rDNA sequences obtained in this study and related species from Genbank (see table 1 for sequence length), was used to produce a pairwise dissimilarity matrix using MEGA 6.0 (Tamura *et al.,* 2011).

Electron microscopy

Pieces of kidney were fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer, pH 7.4 and post-fixed in 1% OsO_4 in cacodylate buffer. Matching kidney material revealed to be positive for malacosporean infection (as identified by PCR and sequencing) was then dehydrated in a graded series of ethanol and embedded in Agar 100 resin (Agar Scientific, Stansted, UK) via propylene oxide. Semi-thin sections were stained with toluidene blue and ultrathin sections with uranyl acetate and lead citrate. Material was examined using a Hitachi H-7650 transmission electron microscope available at the NHM's sister institute, Jodrell Laboratory at Kew Gardens and a LEO 906 electron microscope available at the University of Campinas (UNICAMP), São Paulo, Brazil. We attempted to locate malacosporean infections in at least five kidneys that were identified as positive by PCR of each fish species. The number of kidneys analysed by ultrastructure was ultimately constrained by availability, suitability of material, and time (see Table 3).

Results

Malacosporean infections were detected in the kidney of fishes originating from the UK and Switzerland. Five species were identified: *T. bryosalmonae; Tetracapsuloides* sp. 4 (Bartošová-Sojková *et al*., 2014) (also referred as *Tetracapsuloides* sp. 3 [Patra *et al*., 2016] and from here on

169 called *Tetracapsuloides* sp. 4); *Tetracapsuloides* sp. 5 (Bartošová-Sojková *et al*., 2014) (also
170 referred as *Tetracapsuloides* sp. 2 [Patra *et al*., 2016] and from here on called *Tetracapsuloides* sp.
171 5); *B. plumatellae;* and *Buddenbrockia* sp. 2 (Hartikainen *et al*., 2014) (also referred as
172 *Buddenbrockia* sp. 4 [Patra *et al*., 2016] and from here on called *Buddenbrockia* sp. 2). The
173 infection prevalences of these species ranged from 5.5% to 100% (Table 1).

174 *Tetracapsuloides bryosalmonae* was identified to infect 85.7% of white fish from Lake Lucerne
175 (n = 7), 77.7% of the brown trout specimens examined from the River Stour (n = 9), 89.0% of
176 brown trout from the River Brubach (n = 18), and 100% of rainbow trout from the River Furtbach
177 (n = 12) (Table 1). Young amorphous sporogonic stages (Fig. 1 A) and mature spores (Fig. 1 B-D)
178 of *T. bryosalmonae* were found in kidney tubules of brown trout from the River Stour. Young
179 amorphous sporogonic stages containing sporoplasmosomes with a lucent area (Fig. 1E-F) and a
180 pseudoplasmodium connected to the kidney tubule wall via pseudopodia (Fig. 1 G-H) were
181 observed in white fish from Lake Lucerne. However, because PCR indicated co-infection of this
182 material (see below), it is possible these are immature sporogonic stages of a sphaerosporid.
183 Unfortunately, although five white fish kidneys were examined by ultrastructure, the only kidney
184 that revealed developmental stages was this co-infected material (Table 3).

185 *Buddenbrockia plumatellae* infected 33.3% (n = 16) of the dace specimens from the River Stour,
186 53.8% of the roach from Blickling Lake (n = 13), and 5.5% of the roach from Lake Lucerne (n =
187 18) (Table 1). Unfortunately, ultrastructure was uninformative, being compromised by
188 degeneration of dace material.

189 *Tetracapsuloides* sp. 4 was detected in the kidney of roach sampled from the River Stour at a
190 prevalence of 6.3% (n = 16) (Table 1). Advanced developmental stages anchored to the kidney
191 tubule wall via pseudopodia showed polar capsules and sporoplasmosomes with the characteristic
192 lucent area (Fig. 2A-D).

193 *Tetracapsuloides* sp. 5 was detected in the kidney of gudgeon from the River Stour at a
194 prevalence of 42.9% (n = 21) (Table 1). Clear developmental stages of spores were not observed
195 in kidney tubules.

196 *Buddenbrockia* sp. 2 was detected in 100% (n = 20) of the stone loach specimens sampled from
197 the River Stour (Table 1). Ultrastructural analysis revealed sporogonic stages and mature spores in
198 kidney tubules (Fig. 3 A-E).

199 There was low divergence between the SSU rDNA sequences of the malacosporean species
200 found in this study and the most similar sequences available in Genbank (ranging from 0.1 to 0.3%)
201 (Table 4).

202 Sphaerosporid co-infections were identified in four individuals, two dace from Kent and two
203 white fish from Zurich. Both host species have previously been reported with sphaerosporid
204 infections in Europe (El-Matbouli and Hoffman, 1996; Patra *et al*., 2018). Rounded sporogonic
205 stages (Fig. 4 A and D) with an electron-dense material surrounding each early developmental
206 spore stage (black arrows in Fig. 4 A, B, E, F) were observed in white fish and dace. The same
207 electron-dense material was observed forming the hard valves of a mature spore in white fish (Fig.
208 4 C).

209 Malacosporean infections were detected by ultrastructure for material that was positive by PCR
210 in 4 of 33 cases that were examined (see table 3).

211

212 **Discussion**

213 *Malacosporeans exploit a diversity of fish hosts*

214 Our results demonstrate that a range of fish hosts belonging to different families are used by the
215 two currently recognised malacosporean genera, *Tetracapsuloides* and *Buddenbrockia*. Infection

of trout by *T. bryosalmonae* has been known for decades (Kent and Hedrick, 1985), with many studies demonstrating development in kidney tubules of brown and rainbow trout in the UK and the USA (Kent and Hedrick, 1986; Clifton-Hadley and Feist, 1989; Morris *et al*., 2000; Hedrick *et al*., 1993). *T. bryosalmonae* has been suggested to infect all salmonid species (Hedrick *et al*., 1993) but whether all species serve as effective hosts is unclear. The consistent lack of sporogony in exotic rainbow trout in Europe (Grabner and El-Matbouli, 2008; Hartikainen and Okamura, 2015) demonstrates that, although some salmonids are susceptible to infection, they are accidental hosts. Our results suggest that white fish in Switzerland may also serve as hosts of *T. bryosalmonae* but we were unable to definitively confirm spore production in white fish that were not also infected with sphaerosporids. The prevalences of *T. bryosalmonae* infections were similar in white fish, brown trout and rainbow trout, although it should be stressed that this observation is based on relatively low sample sizes.

Our further studies of *Tetracapsuloides* spp. were also informative. The presence of sporogonic stages including advanced developmental stages of spores of *Tetracapsuloides* sp. 4 in kidney tubules in roach from the River Stour, imply that roach is a true host. The prevalence of infection (6.3%) was lower than that reported in a study based on molecular analyses of roach kidney material from the Czech Republic (100%; Bartošová-Sojková *et al*., 2014). However, the high prevalence reported by Bartošová-Sojková *et al*. (2014) is very likely biased by low sample size (n = 2). *Tetracapsuloides* sp. 5 was detected in kidney of gudgeon from the River Stour where the prevalence of infection was 43.0%. Previous molecular investigation detected this species in gudgeon in the Czech Republic, with prevalences of 33.0 and 91.0% (Bartošová-Sojková *et al*., 2014). We did not observe sporogonic stages in kidney tubules and thus cannot confirm the host status of gudgeon. Nevertheless, recurrent detection of this species in gudgeon (Bartošová-Sojková *et al*., 2014; Patra *et al*., 2016) often at substantial prevalences suggest that infection by *Tetracapsuloides* sp. 5 is common. Further work is required to clarify the host status of gudgeon.

Bartošová-Sojková *et al*. (2014) found *B. plumatellae* infections in dace, roach, and bleak (*Alburnus alburnus*) in the Czech Republic at 100%, 60.0%, and 46.0% infection prevalences, respectively. We detected this species in dace from the River Stour (infection prevalence = 33.3%) and in roach from Blickling Lake (infection prevalence = 53.8%) and Lake Lucerne (infection prevalence = 5.5%). Grabner and El-Matbouli (2010) showed, in a cohabitation study, that *B. plumatellae* was transmitted from bryozoans to carp and minnow. The collective evidence thus suggests that of *B. plumatellae* is able to exploit a range of cyprinid hosts but it remains to be confirmed whether roach and dace support sporogony.

Our detection of *Buddenbrockia* sp. 2 in stone loach is the first time this malacosporean has been linked with a fish host. Hartikainen *et al*. (2014) reported infections of *Buddenbrockia* sp. 2 which develops as myxoworms in the bryozoan *Fredericella sultana* sampled in the UK, Switzerland and Germany. Our ultrastructural analyses revealed sporogonic stages and mature spores in kidney tubules, identifying stone loach as a true host. Thus, the life cycle of *Buddenbrockia* sp. 2 appears to be resolved, with the parasite exploiting *F. sultana* as an invertebrate host and *Barbatula barbatula* as a vertebrate host. It is of course conceivable that further fish and bryozoan hosts may be used.

It should be noted that no signals of kidney infection were observed when fish were dissected to collect material for study, an observation in keeping with the general view that many myxozoan infections are innocuous and/or have little impact on fish hosts (Schulman, 1990; Lom and Dyková, 1992). It is also consistent with the weak or absent immune response, typically observed in natural fish hosts of malacosporeans (Grabner and El-Matbouli, 2008). Such inapparent infections almost certainly contribute to the general lack of investigation of malacosporean infections in fish. Notably, inapparency also characterised the infections of brown trout and white fish by *T. bryosalmonae*, suggesting that environmental conditions and/or fish health status were not

conducive for PKD development. However, the high infection prevalences (78.0% in the River Stour and 87.0% in Lake Lucerne) suggest that many fish may have the potential to develop disease.

We should also note that in only some 12% of cases (4/33) where we obtained positive PCR results were we able to detect malacosporean stages by ultrastructure in the paired kidney material (Table 3). In some cases this was due to degraded material (e.g. dace). In other cases, this could reflect little proliferation and development in kidney, which then made detection by ultrastructure very difficult and eventually we ceased searching.

The challenge of identifying fish hosts

The confirmation of malacosporean fish host status is variously challenging. For example, the lack of detection of malacosporeans in material from Brazil may reflect seasonality, low sample sizes, lack of examination of appropriate fish age classes or absence of bryozoan hosts where the fish were sampled. We anticipate that malacosporeans are present in Brazil in view of observations by Marcus (1941) of *Buddenbrockia* infections in bryozoans in São Paulo State. In addition, ultrastructural detection of sporogonic stages of malacosporeans in the vertebrate host is complicated. It requires extensive sectioning of embedded material to search for small developmental stages, and spores that may be patchily distributed in kidney tubules. The failure to detect sporogonic stages in some cases may simply arise from a limited number of parasites or because the infection has not yet matured. This is compounded by the inapparency of many infections at the macroscopic level.

As shown here, a targeted approach employing associated SSU rDNA sequencing to confirm infection status will at least identify what material to investigate. The alternative approach of conducting transmission trials to confirm that infection is transmitted from fish to bryozoans requires fish husbandry, and permits are often required for such work. We suggest a potential

alternative molecular approach for future identification of fish hosts by determining whether genes specifically involved in polar capsule development (e.g. minicollagens and nematogalactins Holland *et al*., 2011; Shpirer *et al*., 2014, NSPs 1-7 Shpirer *et al*., 2018) are expressed in infected kidney. The rationale is that polar capsules are only present in malacosporean spores and thus the detection of such expressed genes would indicate spore development. In practical terms this would involve preservation of kidney material in e.g. RNAlater and confirmation that these genes are not expressed in pre-sporogonic developmental stages.

Ultrastructural distinction of malacosporean and myxosporean sporogonic stages in fish kidney tubules

We found co-infections of *T. bryosalmonae* and a sphaerosporid species in white fish and of *B. plumatellae* and a sphaerosporid species in dace. The most apparent morphological difference between myxosporean and malacosporean spores is the composition of their valves, which are hardened in myxosporeans but remain soft in malacosporeans (Anderson *et al.,* 1999; Canning and Okamura, 2004). The hardening of myxosporean spore valves is achieved by secretion of chitin (Munoz *et al.,* 1999; 2000; Liu *et al.,* 2011), which may also be associated with internal organelles (Lukeš *et al.,* 1993; Munoz *et al.,* 2000; Redondo *et al.,* 2008). This glycoprotein likely protects myxosporean spores from environmental degradation and maintains spore shape (Munoz *et al.,* 1999; Kaltner *et al.,* 2007; Estensoro *et al.,* 2013). Unprotected malacosporean spores degrade relatively quickly upon release from fish (in <24 h) (de Kinkelin *et al.,* 2002) compared to the chitin-protected spores of myxosporeans. Accordingly, electron microscopy of mature myxosporean spores reveals electron dense valves, and in immature spores an accumulation of electron dense material (inferred to be valve forming material) is observed in the cytoplasm of valvogenic cells (Adriano *et al.,* 2009; Moreira *et al.,* 2014; Morsy *et al.,* 2016). Fibrillar electron dense material reported in valvogenic cells of *Sphaerospora jiroveci* forms a continuous layer

enclosing the developing spore (Dyková and Lom, 1997). Further studies are required to confirm whether this electron dense material is chitin and whether its presence is characteristic of sphaerosporids (Fig. 4B-F).

At least at present, there appear to be no other reliable morphological features that could be used to distinguish between malacosporean and myxosporean developmental stages. For example, although the lucent area of sporoplasmosomes has been highlighted as a malacosporean feature and is evident in the sporoplasmosomes in roach (Figure 2C-D) and white fish (Figure 1E-F), such lucent areas are also occasionally observed in sporoplasmosomes of myxosporeans (Lom *et al.,* 1989; Álvarez-Pellitero *et al.,* 2002; Morris and Freeman 2009; Naldoni, pers. obs.). In addition, the noted tendency for a peripheral distribution of sporoplasmosomes in primary cells of malacosporeans (Canning *et al.,* 2000; Canning *et al.,* 2009) is also sometimes occasionally observed in the primary cytoplasm of myxosporeans (Supamattaya *et al.,* 1993). We would note that in our experience malacosporean sporogonic stages in kidney are difficult to find relative to myxosporean stages. Thus, frequent detection of developmental stages in histological sections could suggest the presence of myxosporeans.

Final comments

Our work has revealed five malacosporeans infecting a variety of fish hosts in the UK and Switzerland, contributing to the growing evidence of a hidden diversity of vertebrate hosts that are exploited by this myxozoan lineage. Further study is necessary to formally describe some of these malacosporean species, to determine if gudgeon act as true hosts of *Tetracapsuloides* sp. 5, to confirm that white fish are true hosts of *T. bryosalmonae*, and to ascertain whether *B. plumatellae* is able to utilise both dace and roach as hosts. It is also clear that the two malacosporean genera are not restricted to exploiting single fish families with *Tetracapsuloides* spp. exploiting members of

Salmonidae and Cyprinidae and *Buddenbrockia* spp. exploiting members of Cyprinidae and Nemacheilidae (Table 1).

Screening both bryozoans and fish has provided vital information about malacosporean diversities and distributions (Bartošová-Sojková *et al*., 2014; Hartikainen *et al*., 2014; Patra *et al*., 2016) and may lead to the resolution of life cycles (this study). Further studies should aim to determine whether malacosporeans generally show broad fish host use or whether some may be specialists. In addition, resolution of accidental hosts and the consequences of dead-end infections could lead to future studies on host-parasite interactions, effective immune responses and the potential dilution of infectious stages when non-permissive hosts are abundant. Finally, resolving malacosporean hosts may help us to understand the range of early hosts that were exploited as cnidarians evolved endoparasitism in view of the primitive nature of malacosporeans (Okamura *et al*., 2015b).

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Reference

Adriano, EA, Arana, S, Carriero, MM, Naldoni, J, Ceccarelli, PS and Maia, AAM (2009) Light, electron microscopy and histopathology of *Myxobolus salminus* n. sp., a parasite of *Salminus brasiliensis* from the Brazilian Pantanal. *Veterinary Parasitology* **165,** 25–29.

Alvarez-Pellitero, P, Molnár, K, Sitjà-Bobadilla, A and Székely, C (2002) Comparative ultrastructure of the actinosporean stages of *Myxobolus bramae* and *M. psuedodispar* (Myxozoa). *Parasitology Research* **88**, 198-207.

Anderson, CL, Canning, EU and Okamura B (1999) Molecular data implicate bryozoans as hosts for PKX (Phylum Myxozoa) and identify a clade of bryozoan parasites within the Myxozoa. *Parasitology* **119**, 555–561.

Atkinson, SD, Bartošová-Sojková, P, Whipps, CM and Bartholomew, JL (2015) Approaches for characterising myxozoan species. In Okamura B, Gruhl A and Bartholomew JL (ed.) *Myxozoan evolution, ecology and development*, Switzerland: Springer International Publishing, pp. 111-123.

Bartošová-Sojková, P, Hrabcová, M, Pecková, H, Patra, S, Kodádková, A, Jurajda, P, Tyml, T and Holzer, AS (2014) Hidden diversity and evolutionary trends in malacosporean parasites (Cnidaria: Myxozoa) identified using molecular phylogenetics. *International Journal for Parasitology* **44,** 565–577.

Bartošová, P and Fiala, I (2011) Molecular evidence for the existence of cryptic species assemblages of several myxosporeans (Myxozoa). *Parasitology Research* **108,** 573–583.

385 **Bucke, D, Feist, SW and Clifton-Hadley, RS** (1991) The occurrence of proliferative kidney
386 disease (PKD) in cultured and wild fish: further investigations. *Journal of Fish Diseases* **14,**
387 583–588.

388 **Canning, EU, Curry, A and Okamura, B** (2009). Early development of the myxozoan
389 *Buddenbrockia plumatellae* in the bryozoans *Hyalinella punctata* and *Plumatella fungosa*, with
390 comments on taxonomy and systematics of the Myxozoa. *Folia parasitologica* **55**, 241-55.

391 **Canning, EU and Okamura, B** (2004) Biodiversity and Evolution of the Myxozoa. *Advances in*
392 *Parasitology* **56,** 43-131.

393 **Canning, EU, Curry, A, Feist, SW, Longshaw, M and Okamura, B** (2000) A new class and
394 order of myxozoans to accommodate parasites of bryozoans with ultrastructural observations on
395 *Tetracapsula bryosalmonae* (PKX organism). *Journal of Eukaryotic Microbiology* **47,** 456-468.

396 **Canning, EU, Curry, A, Feist, SW, Longshaw, M and Okamura, B** (1999) *Tetracapsula*
397 *bryosalmonae* n. sp. for PKX organism the cause of PKD in salmonid fish. *Bulletin of the*
398 *European Association of Fish Pathologists* **19,** 203–206.

399 **Clifton-Hadley, RS and Feist, SW** (1989) Proliferative kidney disease in brown trout *Salmo*
400 *trutta*: further evidence of a myxosporean aetiology. *Diseases of Aquatic Organisms* **6,** 99-103.

401 **de Kinkelin, P, Gay, M and Forman, S** (2002) The persistence of infectivity of *Tetracapsula*
402 *bryosalmonae*-infected water for rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of*
403 *Fish Diseases* **25,** 477–482.

404 **Dyková, I and Lom, L** (1997) Light and Electron Microscope Observations on *Sphaerospora*
405 *ojiroveci* n. sp. (Myxozoa) from the Kidney of *Pangasius sutchi* (Teleostei). *European Journal*
406 *of Protistology* **33,** 444-451.

El-Matbouli, M and Hoffman, RW (1996) Light and electron microscopic descriptions of *Sphaerospora coregoni* El-Matbouli, Hoffmann and Kern, 1995 (Myxosporea: Sphaerosporidae) from the kidney of Whitefish (*Coregonus lavaretus*). *European Journal for Protistology* **32,** 389-398.

Estensoro, I, Álvarez-Pellitero, P and Sijtà-Bobadilla, A (2013) Antigenic characterization of *Enteromyxum leei* (Myxozoa: Myxosporea). *Diseases of Aquatic Organisms* **106**, 149-162.

Feist, SW, Longshaw, M, Canning, EU and Okamura, B (2001) Induction of proliferative kidney disease (PKD) in rainbow trout *Oncorhynchus mykiss* via the bryozoan *Fredericella sultana* infected with *Tetracapsula bryosalmonae*. *Diseases of Aquatic Organisms* **45,** 61–68.

Grabner, DS and El-Matbouli, M (2010) Experimental transmission of malacosporean parasites from bryozoans to common carp (*Cyprinus carpio*) and minnow (*Phoxinus phoxinus*). *Parasitology* **137,** 629–639.

Grabner, DS and El-Matbouli, M (2008). Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) to *Fredericella sultana* (Bryozoa: Phylactolaemata) by various fish species. *Diseases of Aquatic Organisms* **79,** 133–139.

Hartikainen, H, Gruhl, A and Okamura, B (2014) Diversification and repeated morphological transitions in endoparasitic cnidarians (Myxozoa: Malacosporea). *Molecular Phylogenetics and Evolution* **76,** 261–269.

Hartikainen, H and Okamura, B (2015) Ecology and evolution of malacosporean-bryozoan interactions. In Okamura B, Gruhl A and Bartholomew JL (ed.) *Myxozoan evolution, ecology and development*, Switzerland: Springer International Publishing, pp. 201–213.

Hedrick, RP, Baxa DV, de Kinkelin, P and Okamura, B (2004) Malacosporean-like spores in urine of rainbow trout react with antibody and DNA probes to *Tetracapsuloides bryosalmonae*. *Parasitology Research* **92,** 81–88.

Hedrick, RP, MacConnell, E and Kinkelin, P (1993) Proliferative kidney disease of salmonid fish. *Annual Review of Fish Diseases* **3,** 227-290.

Holland, JW, Okamura, B, Hartikainen, H and Secombes, CJ (2011) A novel minicollagen gene links cnidarians and myxozoans. *Proceedings of the Royal Society B* **278,** 546-553.

Kaltner, H, Stippl, M, Knaus, M and El-Matbouli, M (2007) Characterization of glycans in the developmental stages of *Myxobolus cerebralis* (Myxozoa), the causative agent of whirling disease. *Journal of Fish Diseases* **30,** 637−647.

Kent, ML, Andree, KB, Bartholomew, JL, El-Matbouli, M, Desser, SS, Devlin, RH, Feist, SW, Hedrick, RP, Hoffmann, RW, Khattra, J, Hallett, SL, Lester, RJ, Longshaw, M, Palenzeula, O, Siddall, ME and Xiao, C (2001) Recent advances in our knowledge of the Myxozoa. *The Journal of Eukaryotic Microbiology* **48,** 395–413.

Kent, ML and Hedrick, RP (1986) Development of the PKX myxosporean in rainbow trout *Salmo gairdneri*. *Diseases of Aquatic Organisms* **1,** 169-182.

Kent, ML and Hedrick, RP (1985) PKX, the causative agent of Proliferative Kidney Disease (PKD) in Pacific salmonid fishes and its affinities with the Myxozoa. *Journal of Parasitology* **32,** 254–260.

Liu, Y, Zhou, Z, Miao, W, Zhang, Y, Cao, Y, et al (2011) A chitinase from *Aeromonas veronii* CD3 with the potential to control myxozoan disease. *PLoS ONE* **6,** e29091.

Lom, J and Dyková, I (2006) Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitologica* **53,** 1–36.

Lom, J and Dyková, I (1992) Chapter 7 Myxosporidia (Phylum Myxozoa). In Lom, J and Dyková, I (ed.) *Pr*otozoan parasites of fishes, Amsterdam: Developments in Aquaculture and Fisheries Sciences, pp. 159-235.

Lom, J, Feist, SW, Dyková, I and Kepr, T (1989) Brain myxoboliasis of bullhead, *Cottus gobio* L., due to *Myxobolus jiroveci* sp. nov.: light and electron microscope observations. *Journal of Fish Diseases* **12,** 15-27.

Lukeš, J, Volf, P and Lom, J (1993) Detection of chitin in spores of *Myxobolus muelleri* and *M. subepithelialis* (Myxosporea, Myxozoa). *Parasitology Research* **79,** 439-440.

Marcus, E (1941) Sobre Bryozoa do Brasil. Boletim Faculdade de Filosofia, Ciências e Letras, Universidade de São Paulo, *Serie Zoologia* **10,** 3–207.

Medlin, L, Elwood, HJ, Stickel, S and Sogin, ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71,** 491–499.

Moreira, GSA, Adriano, EA, Silva, MRM, Ceccarelli, PS, and Maia, AAM (2014) The morphological and molecular characterization of *Henneguya rotunda* n. sp., a parasite of the gill arch and fins of *Salminus brasiliensis* from the Mogi Guaçu River, Brazil. *Parasitology Research* **113,** 1703-1711.

Morris, D and Freeman, Mark (2009). Hyperparasitism has wide-ranging implications for studies on the invertebrate phase of myxosporean (Myxozoa) life cycles. *International Journal for Parasitology* **40,** 357-69.

Morris, DJ and Adams, A (2006) Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea), the causative organism of salmonid proliferative kidney disease, to the freshwater bryozoan *Fredericella sultana. Parasitology* **133,** 701–709.

Morris, DJ, Adams, A and Richards, RH (2000) Observations on the electron-dense bodies of the PKX parasite, agent of proliferative kidney disease in salmonids. *Diseases of Aquatic Organisms* **39,** 201-209.

Morris, DJ, Adams, A and Richards, RH (1997) Studies of the PKX parasite in rainbow trout via immunohistochemistry and immunogold electron microscopy. *Journal of Aquatic Animal Health* **9,** 265–273.

Morsy, K, Semmler, M, Al-Olayan, E and Mehlhorn, H (2016) *Henneguya collaris* sp. nov., (Myxosporidia), parasite of the Greenband Parrotfish *Scarus collana* Rüppell, 1835 (Actinopterygii, Scaridae) from the Red Sea, Egypt. A light and electron microscopic study. *Parasitology Research* **115,** 2253-2261.

Muñoz, P, Sitjà-Bobadilla, A and Álvarez-Pellitero, P (2000) Ultrastructural localisation of carbohydrates in four myxosporean parasites. *Parasite* **7,** 185−191.

Muñoz, P, Palenzuela, O, Alvarez-Pellitero, P and Sitjà-Bobadilla, A (1999) Comparative studies on carbohydrates of several myxosporean parasites of fish using lectin histochemical methods. *Folia Parasitologica* **46,** 241–247.

Okamura, B, Hartigan, A and Naldoni, J (2018) Extensive uncharted biodiversity: the parasite dimension. *Integrative and Comparative Biology* 1-14. doi: 10.1093/icb/icy039

Okamura, B, Gruhl, A and Bartholomew, JL (2015a) An introduction to myxozoan evolution, ecology and development. In Okamura B, Gruhl A and Bartholomew JL (ed.) *Myxozoan evolution, ecology and development*, Switzerland: Springer International Publishing, pp. 1-20.

Okamura, B, Gruhl, A and Reft, AJ (2015b) Cnidarian origins of the Myxozoa. In Okamura B, Gruhl A and Bartholomew JL (ed.) *Myxozoan evolution, ecology and development*, Switzerland: Springer International Publishing, pp. 45-68.

Patra, S, Bartošová-Sojková, P, Pecková, H, Fiala, I, Eszterbauer, E, and Holzer, AS (2018) Biodiversity and host-parasite cophylogeny of *Sphaerospora* (sensu stricto) (Cnidaria: Myxozoa). *Parasites & Vectors* **11**, 347.

Patra, S, Hartigan, A, Morris, DJ, Kodádková, A and Holzer, AS (2016) Description and experimental transmission of *Tetracapsuloides vermiformis* n. sp. (Cnidaria: Myxozoa) and guidelines for describing malacosporean species including reinstatement of *Buddenbrockia bryozoides* n. comb. (syn. *Tetracapsula bryozoides*). *Parasitology* **144,** 497-511.

Redondo, MJ, Cortadellas, N, Palenzuela, O and Alvarez-Pellitero, P (2008) Detection of carbohydrate terminals in the enteric parasite *Enteromyxum scophthalmi* (Myxozoa) and possible interactions with its fish host *Psetta maxima*, *Parasitology Research* **102**, 1257-1267.

Rey, L (2008) Parasitologia. 4ª edição. Editora Guanabara Koogan.

Shpirer, E, Diamant, A, Cartwright, P and Huchon, D (2018) A genome wide survey reveals multiple nematocyst-specific genes in Myxozoa. *BMC Evolutionary Biology* **18,** 138.

Shpirer, E, Chang, ES, Diamant, A, Rubinstein, N, Cartwright, P and Huchon, D (2014) Diversity and evolution of myxozoan minicollagens and nematogalectins. *BMC Evolutionary Biology* **14,** 205.

Shul'man, SS (1990). Myxosporidia of the USSR. Russian Translations Series 75. A. A. Balkema/Rotterdam.

Supamattaya K, Fischer-Scherl T, Hoffmann RW and Boonyaratpalin S (1993) Light and electron microscope observations on presporogonic and sporogonic stages of *Sphaerospora epinepheli* (Myxosporea) in grouper (*Epinephelus malabaricus*). *The Journal of Eukaryotic Microbiology* **40,** 71-80. doi:10.1111/j.1550-7408.1993.tb04885.x

Tamura, K, Peterson, D, Peterson, N, Stecher, G, Nei, M and Kumar, S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28,** 2731–2739.

Whipps, CM and Kent, ML (2006) Phylogeography of the cosmopolitan marine parasite *Kudoa thyrsites* (Myxozoa: Myxosporea). *Journal of Eukaryotic Microbiology* **53,** 364–373.

Table legends

Table 1. Fish sampled from the UK and Switzerland that were infected with malacosporeans. Details on collection sites, fish hosts, malacosporean species; prevalence of infection, SSU rDNA sequence lengths; sequences that provided the closest match in GenBank, and bryozoans (*) and fishes previously identified to be infected by the respective parasites. Archived samples of brown and rainbow trout known to be infected by *Tetracapsuloides bryosalmonae* (**) or infected by *T. bryosalmonae* through transmission studies (***). bp = base pairs.

Table 2. Fish sampled from the UK, Switzerland, the USA and Brazil in which infections were not detected. Data on collection sites, fish species and the number of fish sampled (No. sampled).

Table 3. The detection of malacosporean infections in fish kidney material from the United Kingdom (UK) and Switzerland (CF) according to PCR, sequencing and ultrastructural analysis. Included are fish species and locality data, the malacosporean species inferred by sequencing, the number (No.) of fish kidneys detected by PCR, the number of kidneys examined by ultrastructure, and the number of kidneys in which spores were detected by ultrastructure. (*) degraded material.

Table 4. Dissimilarity matrix for small subunit ribosomal DNA (SSU-rDNA) of malacosporean species found in this study and their closest matches in Genbank. The upper triangle shows nucleotide differences in relation to the number of bases compared. The lower triangle shows % pairwise distances. SSU rDNA = 18 Small Subunit Ribosomal DNA; UK: United Kingdom, CF: Switzerland. (BS) = Bartošová-Sojková et al. 2014; (H) = Hartikainen et al. 2014.

Figure legends

Fig. 1. Photomicrography of kidney tubules (t) of brown trout collected in the River Stour, Kent, UK, in semi-thin sections stained by toluidine blue (A-B). Transmission electron microscopy (C-H) showing the development of *Tetracapsuloides bryosalmonae* spores in the lumen of the kidney tubules (t) of brown trout (C-D) collected in the River Stour, Kent, UK, and myxozoan development (either malacosporean or sphaerosporid) in white fish (E-H), collected in the Lake Lucerne, Switzerland. (A) Note the presence of two early developmental stages (arrows) developing attached to the kidney tubule wall. Scale bar = 10µm. (B-C) Advanced stage of spore development (empty arrows) showing polar capsule (thin black arrows). Scale bar = 10µm. (D) Mature spore (s) showing polar capsule (empty arrow) with polar filaments (white arrow). Scale bar = 2µm. (E) Primary cell (empty arrow) developing in the lumen of the kidney tubule. Note the presence of sporoplasmosomes (box). Scale bar = 1µm. (F) High magnification of E showing the sporoplasmosomes with a lucent area. Scale bar = 200 nm. (G) Pseudoplasmodium (empty arrow) developing attached to the kidney tubule wall. Scale bar = 10µm. (H) High magnification of G showing pseudoplasmodium connected to the kidney tubule wall via pseudopodia (thin black arrow). Note the two secondary cell nuclei (white arrows). Scale bar = 2µm.

564 **Fig. 2.** Photomicrography of kidney tubules (t) of roach collected in the River Stour, Kent, UK, in
565 semi-thin sections stained by toluidine blue (A), and by transmission electron microscopy (B-D)
566 showing spore development of *Tetracapsuloides* sp. 4. (A) Advanced stage of spore development
567 (empty arrow) with polar capsules (white arrow). Note a stage connected to the kidney tubule wall
568 (thin black arrow). Scale bar = 20µm. (B) Two pseudoplasmodia (p) showing secondary cell nuclei
569 (empty arrows) and a polar capsule (white arrow). Note pseudopodia anchoring the parasite to the
570 kidney tubule wall via pseudopodia (thin black arrows). Scale bar = 2µm. (C) Primary cell (p) with
571 scattered sporoplasmosomes (box). Scale bar = 2µm. (D) High magnification of box in figure C
572 showing details of sporoplasmosomes, each with a lucent area. Scale bar = 500nm.
573
574 **Fig. 3**. Photomicrography of kidney tubules (t) of stone loach collected in the River collected in
575 the River Stour, Kent, UK, in semi-thin sections stained by toluidine blue (A-B), and by
576 transmission electron microscopy (C-E) showing spore development of *Buddenbrockia* sp. 2. (A)
577 Note the different stages of development of spores (arrows) as well as earlier developmental stages.
578 Scale bar = 10µm. (B) Two spores in advanced developmental stages (empty arrows) with polar
579 capsules (black arrows). Scale bar = 10µm. (C) Note two young developmental stages of spores
580 (empty arrows), with a polar capsule in development (large white arrows). Scale bar = 2µm. (D)
581 Pseudoplasmodium (p) in the lumen of the kidney tubule. Scale bar = 5µm. (E) High magnification
582 of D showing pseudoplasmodium connected to the kidney tubule wall via pseudopodia (thin black
583 arrow). Note the secondary cell nucleus (white arrow). Scale bar = 1µm.
584
585 **Fig. 4.** Transmission electron microscopy showing the development of *Sphaerospora* spp. spores
586 in the lumen of kidney tubules of white fish (A-C) collected in the Lake Lucerne, Switzerland and
587 dace (D-F) collected in the River Stour, Kent, UK. (A) Sporogonic stages (empty arrows)
588 developing in the lumen of the kidney tubule of white fish. Scale bar = 10µm. (B) Early sporogonic

stage with electron dense material in valvogenic cells (black arrow) and a mature spore (empty arrow) in the lumen of the kidney tubule. Scale bar = 5µm. (C) High magnification of C showing the mature spore with electron dense material forming the hard valves (black arrow) and two polar capsules (white arrows). Scale bar = 2µm. (D) Sporogonic stages (empty arrows) developing in the lumen of the kidney tubule of dace. Scale bar = 5µm. (E) High magnification of D showing a sporogonic stage with electron dense material in the valvogenic cells (black arrow), the nucleus of the valvogenic cell (nvc), other nuclei (n) and a polar capsule (white arrow). Scale bar = 2µm. (F) A sporogonic stage showing electron dense material in the valvogenic cells (black arrow) and the nucleus of the valvogenic cell (nvc). Scale bar = 1µm.

Table 1.

Country	Water body (latitude; longitude)	Fish species (and Family)	Malacosporean species	Prevalence	SSU rDNA sequence length	SSU rDNA sequence match	Bryozoan* and fishes associated with infection (and author)
United Kingdom	River Stour, Kent (51º13'49" N; 0º58'10" E)	Brown Trout, *Salmo trutta* (Salmonidae)	*Tetracapsuloides bryosalmonae*	77.7% (7/9)	1673 bp	KF731712	*Oncorhynchus mykiss, Salmo trutta* (Bartošová-Sojková *et al*., 2014)
		Dace, *Leuciscus leuciscus* (Cyprinidae)	*Buddenbrockia plumatellae*	33.3% (4/12)	1691 bp	KF731682	*Abramis brama , Alburnus alburnus, Aspius aspius, Blicca bjoerkna, Chondrostoma nasus, Leuciscus idus, Leuciscus leuciscus, Perca fluviatilis, Rutilus rutilus, Scardinius erythrophthalmus, Squalius cephalus* (Bartošová-Sojková *et al*., 2014)
		Roach, *Rutilus rutilus* (Cyprinidae)	*Tetracapsuloides* sp. 4	6.3% (1/16)	1579 bp	KF731727	*Alburnus alburnos, Rutilus rutilus* (Bartošová-Sojková *et al*., 2014)
		Stone Loach, *Barbatula barbatula* (Nemacheilidae)	*Buddenbrockia* sp. 2	100% (20/20)	1693 bp	KJ150268	**Fredericella sultana* (Hartikainen et al., 2014)
		Gudgeon, *Gobio gobio* (Cyprinidae)	*Tetracapsuloides* sp. 5	42.9% (9/21)	1580 bp	KF731729	*Gobio gobio* (Bartošová-Sojková *et al*., 2014)
	Blickling Lake, Norfolk (52º48'45.60" N; 1º13'52.90" E)	Roach, *Rutilus rutilus* (Cyprinidae)	*Buddenbrockia plumatellae*	53.8% (7/13)	1704 bp	KF731682	*Abramis brama, Alburnus alburnus, Aspius aspius, Blicca bjoerkna, Chondrostoma nasus, Leuciscus idus, Leuciscus leuciscus, Perca fluviatilis, Rutilus rutilus, Scardinius erythrophthalmus, Squalius cephalus* (Bartošová-Sojková *et al*., 2014)
Switzerland	Lake Lucerne (47º04'02.72" N; 8º25'53.63" E)	White Fish, *Coregonus albula* (Salmonidae)	*Tetracapsuloides bryosalmonae*	85.7% (6/7)	1683 bp	KF731712	*Oncorhynchus mykiss, Salmo trutta* (Bartošová-Sojková *et al*., 2014)
		Roach, *Rutilus rutilus* (Cyprinidae)	*Buddenbrockia plumatellae*	5.5% (1/18)	1692 bp	KF731682	*Abramis brama , Alburnus alburnus, Aspius aspius, Blicca bjoerkna, Chondrostoma nasus, Leuciscus idus, Leuciscus leuciscus, Perca fluviatilis, Rutilus rutilus, Scardinius erythrophthalmus,*

							Squalius cephalus (Bartošová-Sojková *et al*., 2014)
	River Brubach (47º27'48" N; 9º07'15" E)	** Brown Trout, *Salmo trutta* (Salmonidae)	*Tetracapsuloides bryosalmonae*	88.9% (16/18)	1683 bp	KF731712	*Oncorhynchus mykiss, Salmo trutta* (Bartošová-Sojková *et al*., 2014)
	River Furtbach (47º27'05" N; 8º23'28" E)	***Rainbow Trout, *Oncorhynchus mykiss* (Salmonidae)	*Tetracapsuloides bryosalmonae*	100% (12/12)	1683 bp	KF731712	*Oncorhynchus mykiss, Salmo trutta* (Bartošová-Sojková *et al*., 2014)

Table 2

Country	Site	Fish species (and Family)	N. sampled
United Kingdom	River Stour	Pike, *Esox lucius* (Esocidae)	12
		Perch, *Perca fluviatilis* (Percidae)	14
		Minnow, *Phoxinus phoxinus* (Cyprinidae)	12
		Chub, *Squalius cephalus* (Cyprinidae)	1
	Felbrigg Lake	Pike, *Esox lucius* (Esocidae)	4
Switzerland	Lake Lucerne	Perch, *Perca fluviatilis* (Percidae)	6
		Arctic charr, *Salvelinus alpinus* (Salmonidae)	6
USA	Oklahoma	Paddle fish, *Polyodon spathula* (Polyodontidae)	10
Brazil	Fish farm, Campos do Jordão	Rainbow trout, *Oncorhynchus mykiss* (Salmonidae)	10
	Fish Farm, Santa Cruz da Conceição	Tambaqui, *Colossoma macropomum* (Serrasalmidae)	10
		Pacu, *Piaractus mesopotamicus* (Serrasalmidae)	10
	Lake Santa Fé, Pirassununga	Carp, *Cyprinus carpio* (Cyprinidae)	10
	Amazon River, Santarém	Tambaqui, *Colossoma macropomum* (Serrasalmidae)	3
		Tucunaré, *Cichla monoculus* (Cichlidae)	1
		Caparari, *Pseudoplatystoma punctifer* (Pimelodidae)	1

Table 3.

Fish species and water body	**Malacosporean species**	**No. kidneys positive by PCR**	**No. kidneys examined by ultrastructure**	**No. kidneys with spores**
Brown Trout, *Salmo trutta* River Stour, Kent (UK)	*Tetracapsuloides bryosalmonae*	7	5	1
*Dace, *Leuciscus leuciscus* River Stour, Kent (UK)	*Buddenbrockia plumatellae*	4	4	0
Roach, *Rutilus rutilus* River Stour, Kent (UK)	*Tetracapsuloides* sp. 4	1	1	1
Stone Loach, *Barbatula barbatula* River Stour, Kent (UK)	*Buddenbrockia* sp. 2	20	5	1
Gudgeon, *Gobio gobio* River Stour, Kent (UK)	*Tetracapsuloides* sp. 5	9	5	0
*Roach, *Rutilus rutilus* Blickling Lake, Norfolk (UK)	*Buddenbrockia plumatellae*	7	2	0
White Fish, *Coregonus albula* Lake Lucerne (CF)	*Tetracapsuloides bryosalmonae*	6	5	1
Roach, *Rutilus rutilus* Lake Lucerne (CF)	*Buddenbrockia plumatellae*	1	1	0
*Brown Trout, *Salmo trutta* River Brubach (CF)	*Tetracapsuloides bryosalmonae*	16	3	0
*Rainbow Trout, *Oncorhynchus mykiss* River Furtbach (CF)	*Tetracapsuloides bryosalmonae*	12	2	0
Total	-	**83**	**33**	**4**

Table 4.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1- *T. bryosalmonae* KF731712.1 (BS)	**-**	**5/1673**	**4/1683**	**4/1683**	**2/1683**	39/1726	39/1579	22/1724	23/1580	247/1726	235/1691	235/1692	244/1695	217/1601	223/1693
2- *T. bryosalmonae* (this study - host: brown trout - UK)	**0.3**	-	1/1673	1/1673	2/1673	42/1673	39/1579	25/1673	23/1580	238/1673	236/1668	237/1668	235/1649	219/1601	222/1673
3- *T. bryosalmonae* (this study - host: brown trout - CF)	**0.2**	0.1	-	0/1683	2/1683	41/1683	40/1579	24/1683	24/1580	241/1683	238/1683	239/1683	237/1679	218/1601	212/1683
4- *T. bryosalmonae* (this study - host: rainbow trout - CF)	**0.2**	0.1	0.0	-	2/1683	41/1683	40/1579	24/1683	24/1580	240/1683	237/1683	238/1683	236/1679	218/1601	222/1683
5- *T. bryosalmonae* (this study - host: white fish - CF)	**0.1**	0.1	0.1	0.1	-	41/1683	40/1579	24/1683	24/1580	240/1683	237/1672	238/1672	236/1657	218/1601	222/1671
6- *Tetracapsuloides* sp.4 KF731727 (BS)	2.3	2.5	2.4	2.4	2.4	-	**1/1579**	41/1724	41/1580	243/1725	231/1691	231/1692	240/1695	216/1601	222/1693
7- *Tetracapsuloides* sp.4 (this study - host: roach - UK)	2.5	2.5	2.5	2.5	2.5	**0.1**	-	41/1579	40/1577	225/1579	226/1579	226/1579	226/1579	217/1577	218/1579
8- *Tetracapsuloides* sp.5 KF731729 (BS)	1.3	1.5	1.4	1.4	1.4	2.4	2.6	-	**1/1580**	239/1724	227/1691	2227/1692	236/1695	218/1601	224/1693
9- *Tetracapsuloides* sp.5 (this study - host: gudgeon - UK)	1.5	1.5	1.5	1.5	1.5	2.6	2.5	**0.1**	-	222/1580	223/1580	223/1580	223/1580	219/1580	220/1580
10- *B. plumatellae* KF731682 (BS)	14.5	14.5	14.6	14.5	14.5	14.3	14.5	14.1	14.3	-	**1/1691**	**1/1692**	**2/1695**	67/1601	70/1693
11- *B. plumatellae* (this study - host: dace - UK)	14.3	14.4	14.5	14.4	14.4	14.1	14.6	13.8	14.4	**0.1**	-	1/1691	1/1666	68/1601	69/1691
12- *B. plumatellae* (this study - host: roach - CF)	14.3	14.4	14.5	14.5	14.5	14.1	14.6	13.8	14.4	**0.1**	0.1	-	2/1666	68/1601	70/1692
13- *B. plumatellae* (this study - host: roach - UK)	14.2	14.5	14.5	14.5	14.5	14.6	14.6	14.3	14.4	**0.1**	0.1	0.1	-	69/1601	70/1693
14- *Buddenbrockia* sp. 2 KJ150268 (H)	14.0	14.1	14.0	14.0	14.0	13.9	14.0	14.0	14.1	4.2	4.3	4.3	4.3	-	**5/1601**
15- *Buddenbrockia* sp. 2. (this study - host: loach - UK)	13.5	13.5	13.5	13.5	13.5	13.5	14.0	13.6	14.1	4.2	4.1	4.2	4.2	**0.3**	-

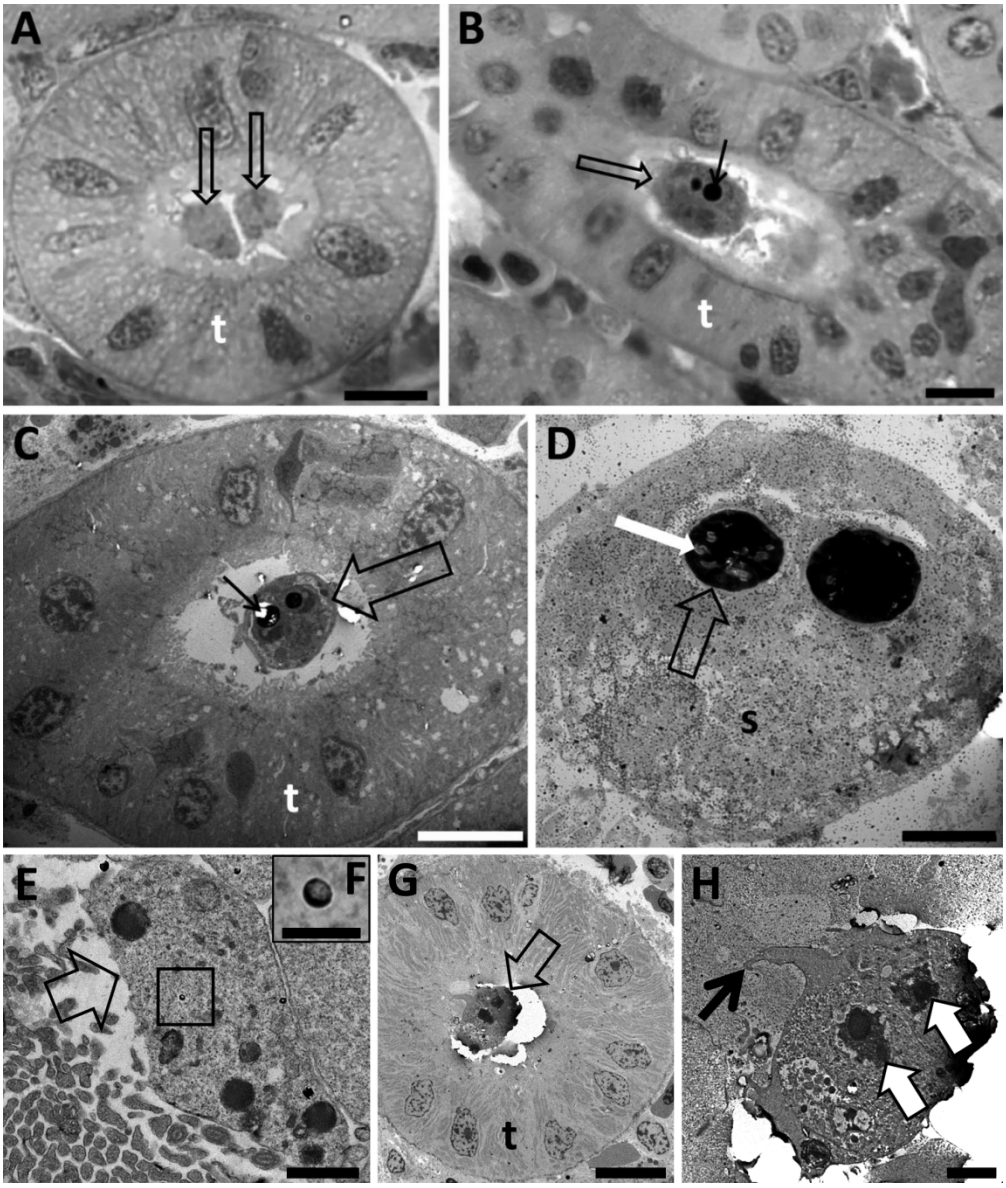

Fig. 1. Photomicrography of kidney tubules (t) of brown trout collected in the River Stour, Kent, UK, in semi-thin sections stained by toluidine blue (A-B). Transmission electron microscopy (C-H) showing the development of Tetracapsuloides bryosalmonae spores in the lumen of the kidney tubules (t) of brown trout (C-D) collected in the River Stour, Kent, UK, and myxozoan development (either malacosporean or sphaerosporid) in white fish (E-H), collected in the Lake Lucerne, Switzerland.

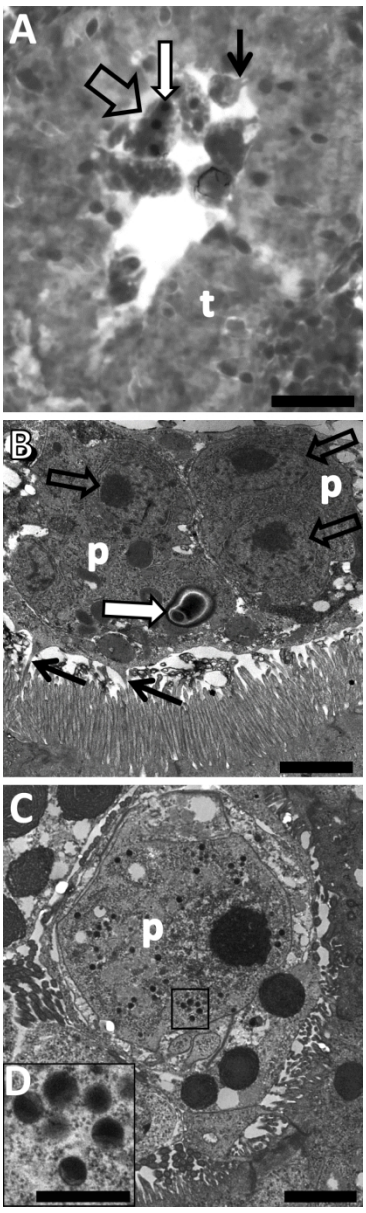

Fig. 2. Photomicrography of kidney tubules (t) of roach collected in the River Stour, Kent, UK, in semi-thin sections stained by toluidine blue (A), and by transmission electron microscopy (B-D) showing spore development of Tetracapsuloides sp. 4.

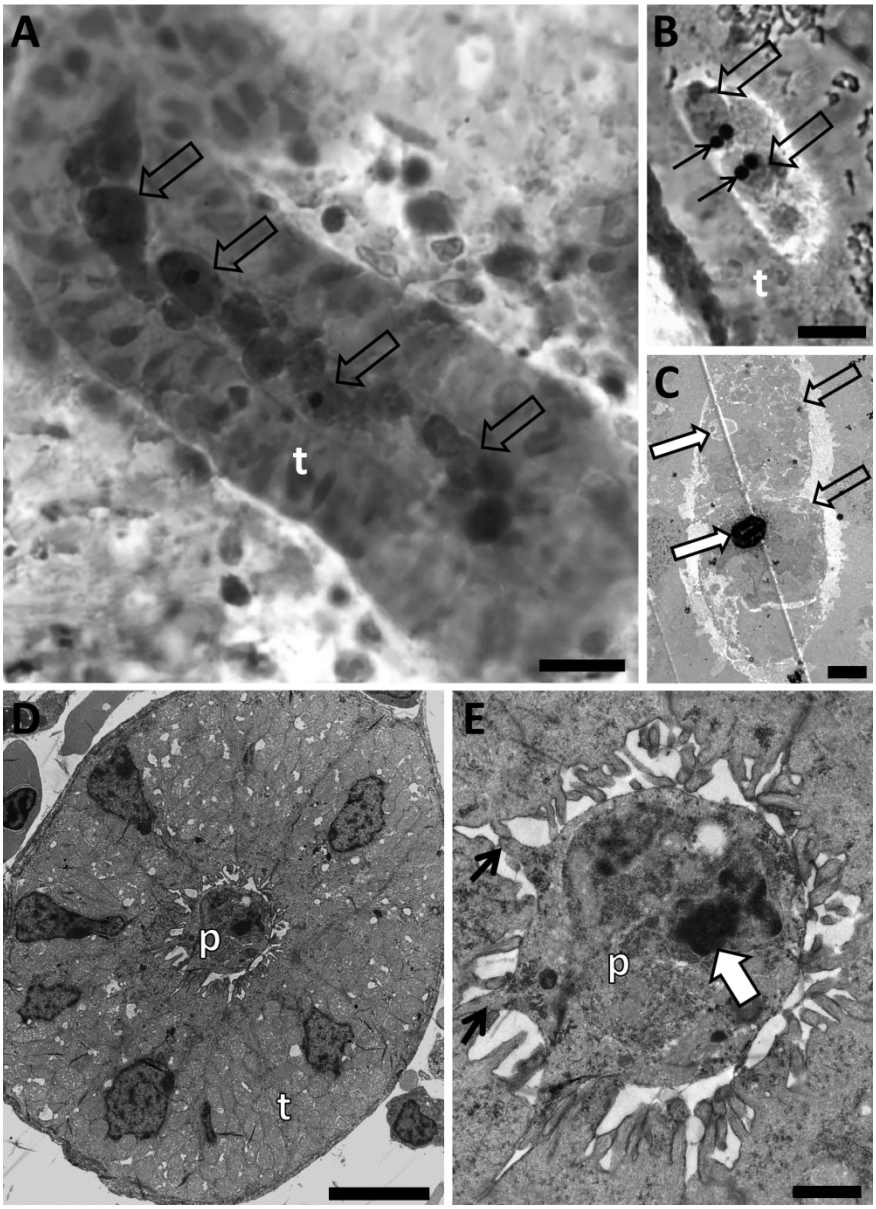

Fig. 3. Photomicrography of kidney tubules (t) of stone loach collected in the River collected in the River Stour, Kent, UK, in semi-thin sections stained by toluidine blue (A-B), and by transmission electron microscopy (C-E) showing spore development of Buddenbrockia sp. 2.

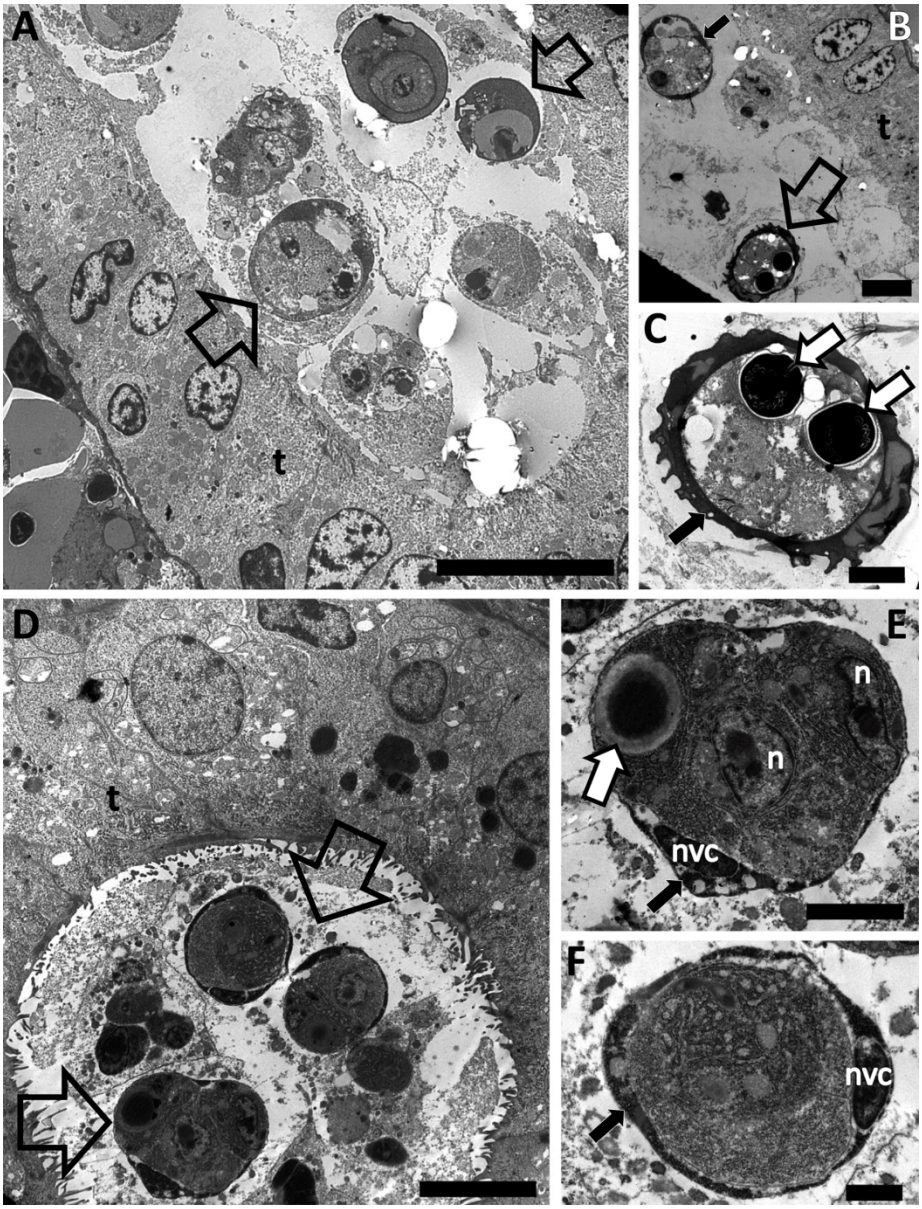

Fig. 4. Transmission electron microscopy showing the development of Sphaerospora spp. spores in the lumen of kidney tubules of white fish (A-C) collected in the Lake Lucerne, Switzerland and dace (D-F) collected in the River Stour, Kent, UK.

Address to the Editor Date: February 13rd 2019

Dr. Stephen Phillips

Parasitology

Dear Editor,

We would like to thank you for the opportunity of resubmitting a revised version of the manuscript MS ID: PAR-2018-0350

Title: Malacosporean myxozoans exploit a diversity of fish hosts.

Below we describe how we have dealt with the various points raised by the reviewers and yourself.

Sincerely yours,

Dr. Juliana Naldoni, on behalf of all co-authors

Editor Comments

The referees find much of value in your manuscript, but they also raise a number of points that require your attention (specific comments from the referees are appended to the foot of this email). Please note: if your original submission did not include continuous line numbering, we will have added this to make the referees' job easier, and may also have undertaken some basic formatting. Therefore, you should use the version of your paper currently available on Scholar One for your revision.

I invite you to submit a revised manuscript that addresses these points. However, you will understand that this will have to be refereed again and, at this stage, I can give no assurance of subsequent acceptance.

Response: We would like to thank the reviewers and the editor for their time in evaluating our submission and for their helpful comments. We believe that the revised version satisfactorily addresses the referees' various questions and concerns. Below we provide our responses following the various referees' comments. In addition we have highlighted

all changes to our manuscript in red. Thank you again for your time and efforts on our manuscript.

Referee: 1

Comments to the Author

MS nr. PAR-2018-0350

Malacosporean myxozoans exploit a diversity of fish hosts

Overall, I liked the manuscript, especially the success of the authors to prove several fish hosts as true hosts for the development of previously identified malacosporean species and thus aid to the knowledge of the biodiversity and host spectra of this fascinating parasite group.

The English language is of very good level and no corrections are needed.

Content wise, I have several points that require authors attention:

Lines 77-85: Regarding fish as putative accidental hosts, I would add a point that the malacosporean blood stages may have been detected in PCR positive fish though they may not finish their development in those fish hosts.

Response: We have added this detail in lines 84-85 as:

"…malacospororeans could invade fish as blood stages that are detected by PCR of fish kidney but fish may not support subsequent development."

Lines 121-123: Why the published PCR conditions (Grabner and El-Matbouli 2010) have been modified for budd-f/budd-r primer combination? Did the authors aim at increasing the primer specificity? Include the explanation in the methods section.

Response: The original cycling conditions were modified with the aim of increasing the primer specificity (lines 132-133).

Lines 139-140: Looking at Table 3, the authors rather present pairwise dissimilarity matrix. Specify how long was the final alignment used for the matrix and if the original alignment was used or if some alignment editing was done.

Response: We modified to describe as a "dissimilarity matrix". Alignment editing was not performed. Table 3 is now Table 4 and it has been revised and the number of

nucleotides compared (i.e. how long the original alignment was) is now shown in the table.

Line 174: What is the proof these are mature spores? The polar capsules are not visible due to poor quality of the slide. I recommend omitting Fig. 2 D-E.

Response: Figure 2 was modified and Fig. 2A now shows spores with polar capsules developing in advanced developmental stages of spores (lines 190-192).

Line 184: Did the authors aim at sequencing the sphaerosporid species? If yes, what was their status?

Response: Both hosts have been previously identified with sphaerosporid infections in Europe (Patra et al. 2018 and El-Matbouli and Hoffman 1996) confirming that our positive PCR for sphaerosporid infections was not surprising. Our aim was to characterise malacosporeans by sequencing and ultrastructure (not sphaerosporids).

Line 209: I would not mention mature spores for what the authors have observed for Tetracapsuloides sp. 4 as their TEM findings do not prove this. See point for line 174.

Response: A new Fig. 2A was inserted and it shows spores with polar capsules, and advanced developmental spore stages (lines 190-192 and 229).

Lines 211-212: 100% prevalence of Tetracapsuloides sp. 4 from roach is very likely biased by low sample size in the Bartošová-Sojková et al. (2014) study.

Response: A reference to this bias has now been included in the manuscript (lines 232-234).

Line 250-251: It may also reflect the absence of favorable conditions for the malacosporean life cycles, e.g. for the growth of bryozoan hosts.

Response: A good point – this has been inserted in the manuscript (lines 276-277).

Lines 266-268: It is generally a great idea but may bring several obstacles if not done with caution. I believe it is sufficient to say like formulated in the MS but if the authors plan to perform this research in the future a detailed preliminary study on the development of the target gene(s) in different developmental stages of a malacosporean is required to have an overall idea if those genes are not expressed before spore formation. In the case some (even if low) expression is encountered in the pre-sporogonic stages, the threshold has to be wisely defined to make sure that particular expression relates to the presence of spore stage(s).

Response: Thank you for drawing our attention to these points for future research.

Lines 270-283: I feel this paragraph needs particular attention as several statements are not sufficiently referenced or are not correct. In detail:

Line 275 (chitin): Include more references, e.g. Lukeš et al. (1993), Estensoro et al. 2013.

Line 277: "The soft valves of malacosporean spores lack chitin". I believe it is very probably like the authors say but unfortunately I don't see any statement about this in the cited paper. The optimal approach would be to base such statement on an experimental study focused on examining such differences in spore structure. Please direct the reviewer to a specific part of the cited paper where chitin is mentioned or provide an alternative reference.

Response: The above related comments are particularly helpful and we have revised the entire paragraph to clarify chitin production and distribution in myxosporeans and malacosporeans, adding various references (lines 299-316).

Line 279: "Sphaerosporid stages are not attached". This is not true and if no difference in the attachment strategy is found, it has to be excluded from the differential "diagnosis" of malacosporean and sphaerosporid sporogonic stages, as there are several good examples of Sphaerospora pseudoplasmodia attached to the host tissue cells: e.g. Jirků and Bartošová-Sojková (2014), Lom et al. 1985 , Lom e al. 1991, possibly Chen et al. 2010 (in DAO)...

Response: We have revised the paragraph and have now excluded attachment of sphaerosporids as helping with differential “diagnosis”.

Lines 280-281: "These organelles (that) are lacking in sphaerosporids". This has to be reformulated to be more accurate. Sphaerosporids are not lacking these organelles - they are present in the sporoplasm cytoplasm of every myxosporean, however, they have not been observed in the primary cells of sphaerosporids unlike in malacosporeans. Important trait may be that sporoplasomosomes of malacosporeans and myxosporeans differ in their location and also ultrastructurally (Canning et al. 2000, 2002, 2007; Morris and Adams 2006, 2007) - in the lack of the central lucent area in myxosporean sporoplasmosomes.

Response: We have undertaken further examination of the literature and other evidence which leads us to believe that sporoplasmosomes are not going to be useful features for distinguishing malacosporeans from sphaerosporids. (lines 317-328).

Lines 281-283: This is a very vague statement and needs to be confirmed by some study that clearly proves that the electron dense material within the pseudoplasmodia relates to a sphaerosporid development. If existing, provide a reference.

Response: We have revised this section and provided references (lines 299-316).

Regarding all pictures: Do the pictures of malacosporean stages originate from samples with single species infection to make sure the youngest stages are not misidentified with sphaerosporids?

Response: Only the images of white fish originate from samples infected with both *T. bryosalmonae* and a sphaerosporid species (lines 223-225). All the other pictures of malacosporean stages originate from samples with single species infection. Because we are now not confident that the sporoplasmosome arguments support our interpretation that this material is definitely *T. bryosalmonae* we have revised the text to say that confirmation of white fish as true hosts of *T. bryosalmonae* is required.

Figs. 1 and 3: I recommend to unify the colour of the semithin and ultrathin sections in black and white.

Response: This has been done.

Fig. 1D: What is labeled by S - not present in the legend.

Response: It was inserted in the legend (line 556).

Fig. 1E and 2A: Did the authors observe sporoplasmosomes in the primary cells on these figures? If not, how do they explain this fact?

Response: Figure 1E was replaced and the sporoplasmosomes are clearly seen in the primary cell shown in Fig.1E-D. Figure 2A was also replaced and the organization of the figure was modified. The sporoplasmosomes are now identified in the primary cell cytoplasm shown in Fig. 2C-D and commented on in the manuscript (lines 317-328).

Fig. 2. "...kidney tubules (t)..." I dont see a "t" label in any part of this figure.

Response: The “t” is now in the new Fig. 2A.

Table 1, last column: I would somehow distinguish the bryozoan and fish species.

Response: The distinction is now made by using an asterisk to distinguish bryozoan host in Table 1.

Table 3: This is a dissimilarity matrix. "18 Small Subunit Ribosomal DNA" - why all in capitals?

Response: This has been modified (line 541).

Referee: 2

Comments to the Author

Naldoni and colleagues present data on abundance of malacosporean parasites in multiple fish species from fish in rivers in England and Brazil, and England). This base-level diversity and abundance data are important for this group of parasite, as fewer than 20 species are presently known, compared with closely-related myxosporeans, which are diverse and speciose (with probably many thousands of species).

This is a well-written paper and I have no hesitation recommending that it be published. I found only a few typographical errors (see line-specific comments below). I have a few comments about the methods and results, but these border on rhetorical and though they should be considered by the authors, they are not significant in terms of the publishability of the paper.

The most significant finding of the present study was that they did not find any additional malacosporeans, despite sampling novel fish species from diverse rivers (UK, Switzerland, Brazil, USA). The malacosporean species they detected by DNA sequence identity (and in some cases were able to describe with electron microscopy) were all observed previously in fish in mainland Europe. The other significant negative result is that no species were described in Brazil (where none are known), despite abundant myxosporean parasites there. These low/negative results inform our fundamental understanding of malacosporean evolution. This probably indicates constraints to malacosporean dispersal and diversity given the essential bryozoan host in all malacosporean life cycles (as opposed to myxosporeans and their annelid hosts). Naldoni et al., also demonstrate the effectiveness (albeit numerically challenging) to use EM to verify parasite development in PCR-positive fish, and thereby screen out incidental DNA-only detections.

A few questions regarding PCR approach: how general are the PCR primers you used for malacosporeans? Do you have any hypothesis or data on the rate that you may have "missed" finding malacosporeans, given that only PCR-positive fish were examined by EM…(this is a rhetorical questions I realize). I was wondering if your lack of diversity is an artifact of primer bias (i.e. more divergent malacosporeans were not amplified, and therefore missed?). Did you consider (or would you propose) a subsample of PCR-negative fish be checked by EM? (though I appreciate the points you make L252-256). I suggest the simplest approach might be to use more general primers on the kidney samples, and sequence every positive, maybe pairing known malaco-specific primers with more general myxo primers. PCR is the cheapest/easiest part of the methods presented, and could be expanded in future studies.

Response: We are grateful for the comments from this reviewer. The primers we used specifically amplify only malacosporean species so they are specific at this taxon level, detecting DNA of taxa of the two known genera (*Tetracapsuloides* and *Buddenbrockia*).

We adopted this approach as we were specifically interested in malacosporean diversity and not diversity of other myxozoans. We cannot ensure that false negative PCRs did not happen, although the literature has shown that the primers MalaF and MalaR, previously used to detect positive samples are efficient. We have added that we trialled more general primers and that they did not work to detect malacosporean infection (lines123-124). We did observe that for many positive PCR samples malacosporean stages were not observed by ultrastructure. This is an important point that we overlooked because future researchers might be interested in how much effort is potentially involved in finding developmental stages by ultrastructure. We have now mentioned this in the Material and Methods (lines 161-163), Results (lines 209-210), and have summarised the data in Table 3. We have again referred to this issue in our Discussion section (lines 267-271). Theoretically, we could have looked at all material fixed for TEM analysis even that which was negative for malacosporean infection by PCR analysis. This, however, was highly impractical in view of cost and time as is now much more clear by providing Table 3 and thus demonstrating that finding spore development in kidneys already known as positive for malacosporean infection is difficult.

I found only a few minor text errors or things I have to query:

L96 What guided fish sampling? i.e. were they targeted for some reason? Or just as available? Please add a line explaining what fish and why.

Response: An explanation has been added in lines 98-103.

L128-L131 there are mis-matched square brackets.

Response: This has been corrected (lines 137-140).

L160-163 inconsistent numbers of decimals (e.g. 77.7% versus 89%)

Response: This has been corrected in the whole manuscript.

L182 "ranging from" not "ranging of"

Response: Corrected (line 200).

L354 "DS" not "DS."

Response: Corrected (line 416).

L415 Is "144(04)" correct?

Response: This has been corrected (line 502).