Vol. 127: 151–156, 2018
https://doi.org/10.3354/dao03200

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published January 31

NOTE

Tetracapsuloides bryosalmonae persists in brown trout *Salmo trutta* for five years post exposure

Hatem Soliman*,, Gokhlesh Kumar**, Mansour El-Matbouli**

Clinical Division of Fish Medicine, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

ABSTRACT: *Tetracapsuloides bryosalmonae* is a malacosporean parasite and the causative agent of proliferative kidney disease (PKD) that seriously impacts farmed and wild salmonids. The parasite's life cycle includes an invertebrate host, the bryozoan *Fredericella sultana*, and a vertebrate host, salmonid fish. The persistence of *T. bryosalmonae* in brown trout *Salmo trutta* for up to 2 yr following exposure is well documented. Results from the present study confirmed that one brown trout that had recovered from PKD did not completely clear the parasite from its tissues and that *T. bryosalmonae* could persist in brown trout for up to 5 yr post exposure. Furthermore, recovered infected brown trout can release viable *T. bryosalmonae* spores that are able to infect specific pathogen-free *F. sultana* colonies. *T. bryosalmonae* DNA was detected by PCR in every organ, and parasite stages were observed in the kidney, spleen and liver following immunohistochemistry. This finding indicates that *T. bryosalmonae*-infected brown trout can act as asymptomatic carriers and release the parasite for several years after the initial infection, acting as a reservoir of infection, and contributing to the dissemination of the parasite to new areas.

KEY WORDS: Malacosporean · Proliferative kidney disease · *Fredericella sultana* · Persistence of infection

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Proliferative kidney disease (PKD) is a significant parasitic disease that impacts the trout industry in Europe and North America (Hedrick et al. 1993, El-Matbouli & Hoffmann 2002). The disease is caused by the myxozoan *Tetracapsuloides bryosalmonae* that affects both farmed and wild fish species, and the decline of wild salmonid populations in several rivers has been attributed to PKD (Feist et al. 2002, Wahli et al. 2007, Skovgaard & Buchmann 2012, Carraro et al. 2016, Mo & Jørgensen 2017). Surveys have shown that *T. bryosalmonae* is widespread in Swiss rivers (Wahli et al. 2007) as well as in the rivers of southern England (Fontes et al. 2017). PKD has been associated with high morbidity in farmed fish and mortalities that can reach 100% due to the secondary infection (Feist & Bucke 1993). Abd-Elfattah et al. (2014a) reported that vertical transmission allows *T. bryosalmonae* to persist in the bryozoan host. Gorgoglione et al. (2016) reported that migrating zooids were able to escape from deteriorating bryozoan colonies and suggested that this represented a way through which *T. bryosalmonae* could spread to new habitats.

The life cycle of *T. bryosalmonae* alternates between invertebrate and vertebrate hosts, the bryozoan *Fredericella sultana* and salmonid fish, respectively (Morris & Adams 2006, Grabner & El-Matbouli 2008, Okamura et al. 2011). A single spore is sufficient to infect a fish and cause clinical symptoms of PKD (McGurk et al. 2006). Spores of *T. bryosalmonae* released from infected bryozoan colonies infect fish through the gills where they enter the circulatory

*Corresponding author: hatem.soliman@vetmeduni.ac.at
**These authors contributed equally to this work

© Inter-Research 2018 · www.int-res.com

system and migrate to the kidney where the parasite undergoes extra-sporogonic multiplication in the interstitium and differentiation through sporogenesis in the kidney tubules (Grabner & El-Matbouli 2008). Mature spores are excreted via urine in the environment where they can infect bryozoan colonies and complete their life cycle (Hedrick et al. 2004, Morris & Adams 2006, Grabner & El-Matbouli 2008).

Analysis of internal transcribed spacer sequence data from *T. bryosalmonae* confirmed the existence of 2 lineages of *T. bryosalmonae*: the European lineage and the North American lineage (Henderson & Okamura 2004). European *T. bryosalmonae*-infected brown trout *Salmo trutta* release viable spores that are infective to their invertebrate bryozoan host (Morris & Adams 2006) and can complete the life cycle. According to Grabner & El-Matbouli (2008) and Kumar et al. (2013a) rainbow trout *Oncorhynchus mykiss* infected by European *T. bryosalmonae* cannot release viable spores of *T. bryosalmonae*. The spores of the North American *T. bryosalmonae* have been observed in the urine of infected rainbow trout (Hedrick et al. 2004).

Water temperature plays an important role in the development of *T. bryosalmonae* in its bryozoan host as well as in the development of clinical signs and mortality in the fish (Bettge et al. 2009). PKD-related clinical signs and fish mortality increase with increasing water temperature; accordingly, the distribution and severity of PKD are expected to increase in the open water due to climate change (Hedrick et al. 1993, Tops et al. 2006, Okamura et al. 2011). Clinical signs and lesions associated with PKD include pale gills due to anemia, lateral body swelling, exophthalmia and splenomegaly. The kidney is often grey, mottled and uniformly enlarged (Hedrick et al. 1993). The persistence of PKD infection in brown trout for up to 2 yr post exposure and the ability to release mature spores was reported by Abd-Elfattah et al. (2014b).

The aim of the present study was to investigate the persistence and the infectivity of *T. bryosalmonae* in infected brown trout 5 yr post exposure.

MATERIALS AND METHODS

Fish

The present experiment is the extension to the experiment that started on 5 January 2012 and is described in detail by Abd-Elfattah et al. (2014b). Briefly, specific pathogen-free (SPF) brown trout were obtained as eyed eggs from a certified Austrian hatchery and maintained in our SPF facility. Prior to infection, 5 SPF brown trout were sampled randomly and tested for the presence of *Tetracapsuloides bryosalmonae* by PCR (Grabner & El-Matbouli 2009). On 5 January 2012, 25 brown trout (mean length 5.4 ± 0.5 cm, weight 2.6 ± 0.5 g) were cohabitated for 2 d with *T. bryosalmonae*-infected *Fredericella sultana* colonies and maintained at 16 ± 2°C (in the winter 14 to 16°C and in the summer 16 to 18°C) under controlled laboratory conditions. A control group with the same number of brown trout was cohabitated for 2 d with SPF *F. sultana* colonies from our laboratory stock and maintained at 16 ± 2°C. In a previous study, 24 fish were sampled at different time points up to 104 wk post exposure to verify the infectivity of the released *T. bryosalmonae* spores and the results of these samples were published by Abd-Elfattah et al. (2014b). One fish has remained from this experiment; this fish recovered from the infection and displayed no clinical signs. This remaining fish was maintained for 5 yr post exposure under the same conditions to investigate how long *T. bryosalmonae* spores can persist in brown trout and release spores that remain infective for the bryozoan host.

Cohabitation of SPF *F. sultana* colonies to the infected brown trout

SPF *F. sultana* colonies were cultivated in our laboratory and fed with algae according to Abd-Elfattah et al. (2014b). Briefly, free statoblasts were dissected from our SPF laboratory-cultured *F. sultana* colonies, and then these colonies were examined under a microscope and subject to PCR to test the presence of any *T. bryosalmonae* infection (Grabner & El-Matbouli 2009). The collected statoblasts were incubated in Bryozoan Medium C (BMC) in Petri-dishes at 4°C for 2 wk in the dark and then at 15°C for 1 wk (12 h light, 12 h dark). They were then transferred to 2 l containers filled with BMC and kept at 18°C with slow aeration and illumination (Kumar et al. 2013b). The hatched statoblasts were examined under a microscope and subjected again to PCR (Grabner & El-Matbouli 2009) to confirm absence of the *T. bryosalmonae* infection.

Cohabitation of SPF *F. sultana* colonies with the remaining infected brown trout was done following the protocol described by Kumar et al. (2013b): The bryozoans colonies were placed alongside the fish for

8 h d^{-1} over a 2 wk period and water circulation was stopped for the duration of the cohabitation. Cohabitated *F. sultana* colonies were examined weekly by stereomicroscope for development of *T. bryosalmonae* sacs and subjected to PCR according to Grabner & El-Matbouli (2009) to confirm the presence of the parasite.

Immunohistochemistry and PCR investigations

At the end of the 5 yr period, the maintained brown trout was euthanized by prolonged immersion in a solution of Tricaine methanesulfonate (MS-222, Sigma Aldrich) at a concentration of 0.1 g l^{-1}. A standard necropsy was performed and the fish was assessed for macroscopic changes typical of PKD infection. Kidney, liver, spleen, heart, gills, intestine and brain were sampled. Tissue samples were cut into 2 parts; one part used for DNA extraction and the other part was fixed in 10% buffered formalin for immunohistochemistry investigation. DNA was extracted from each tissue sample using a QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer's instructions. Nested PCR was carried out for the detection of *T. bryosalmonae* with primers 5F (5′-CCT ATT CAA TTG AGT AGG AGA-3′) and 6R (5′-GGA CCT TAC TCG TTT CCG ACC-3′) according to Kent et al. (1998) in a first round, followed by a second PCR round with primers PKD-real F (5′-TGT CGA TTG GAC ACT GCA TG-3′) and PKD-real R (5′- ACG TCC GCA AAC TTA CAG CT-3′) according to Grabner & El-Matbouli (2009). The nested PCR products (166 bp) were purified using MinElute Gel Extraction Kit (QIAGEN) and then cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's instructions. Purified plasmids were sequenced in a commercial sequencing laboratory (LGC Genomics) and then compared with the *T. bryosalmonae* 18S rDNA gene sequence (GenBank accession number U70623).

Immunohistochemical staining was performed according to Kumar et al. (2013b). Briefly, the fixed fish organs (kidney, spleen, liver, intestine, heart, gills and brain) in 10% neutral buffered formalin were washed, dehydrated and embedded in paraffin. The species-specific P01-*Tetracapsuloides bryosalmonae* (PKX) monoclonal antibody (Aquatic Diagnostics) was used for immunohistochemically staining of the 5 µm tissue sections according to the manufacturer's instruction. Subsequently, a Dako EnVision+ Kit (HRP; AEC) was used for visualization of the antigen–antibody reaction on the tissue sections. Sections were counterstained with hematoxylin, mounted and examined for parasite stages under a microscope.

RESULTS

Specific pathogen-free *Fredericella sultana* colonies that cohabitated with *Tetracapsuloides bryosalmonae*-infected brown trout showed the characteristic parasite mature sacs floating in the bryozoans' metacoel within 6 to 8 wk post exposure. The parasite sacs were confirmed to be *T. bryosalmonae* based on microscopic examination (Fig. 1) and PCR (data not shown).

At the time of termination, the 5 yr old brown trout was 35 cm in length and weighed 511 g. Clinical examination revealed the absence of any clinical signs related to either proliferative kidney disease (Fig. 2) or any other infection or infestations. During necropsy, no internal PKD lesions such as swollen kidney, splenomegaly or pale liver, were observed.

The nested PCR assays resulted in the amplification of the expected 166 bp fragment of *T. bryosalmonae* from every organ samples tested (kidney, liver, spleen, heart, gills, intestine and brain). Sequences of the nested PCR products revealed 100% similarity to the *T. bryosalmonae* 18S rDNA gene (GenBank accession number U70623).

Immunohistochemistry staining revealed low numbers of intra-luminal sporogonic stages of *T. bryosalmonae* in the kidney (Fig. 3A), with very low numbers of pre-sporogonic stages. Additionally, low

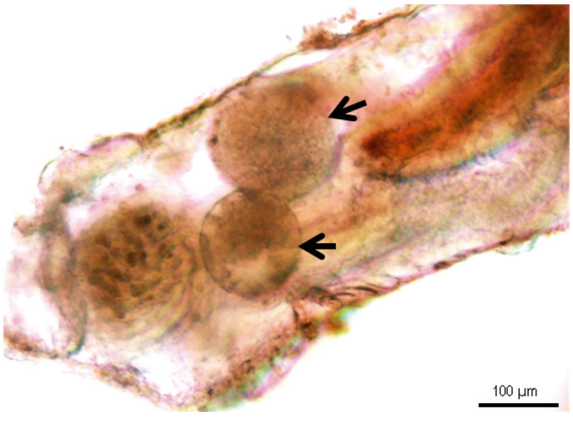

Fig. 1. *Fredericella sultana* colony infected with *Tetracapsuloides bryosalmonae*. Two mature spore sacs (arrows) are visible in the body cavity of *F. sultana* following cohabitation with a brown trout *Salmo trutta*, 5 yr after the fish had been infected

Fig. 2. Brown trout *Salmo trutta*, 5 yr post exposure to *Tetracapsuloides bryosalmonae*. No abnormal clinical signs were observed on the fish

numbers of pre-sporogonic parasite stages were observed in the spleen and liver tissues (Fig. 3B,C). No pre-sporogonic parasite stages were observed in the intestine, heart, gills and brain tissues.

DISCUSSION

The present work reports the results of a long-term experiment regarding the persistence of *Tetracapsuloides bryosalmonae* in brown trout: The initial exposure of the brown trout to *T. bryosalmonae* was performed in January 2012 and the initial result of this experiment confirmed the persistence of the parasite in the chronically infected brown trout for 2 yr (Abd-Elfattah et al. 2014b). One chronically infected brown trout that had recovered from the infection was kept from this initial experiment to infect *Fredericella sultana* colonies per cohabitation as well as to investigate for how long *T. bryosalmonae* can persist in the infected brown trout and release viable *T. bryosalmonae* spores. The maintained brown trout was releasing viable *T. bryosalmonae* spores that were infective to the *F. sultana* colonies for more than 5 yr post exposure, up to the date of the experiment's termination on March 2017.

Moreover, *T. bryosalmonae* DNA was detected in all organs tested by PCR and low numbers of parasite

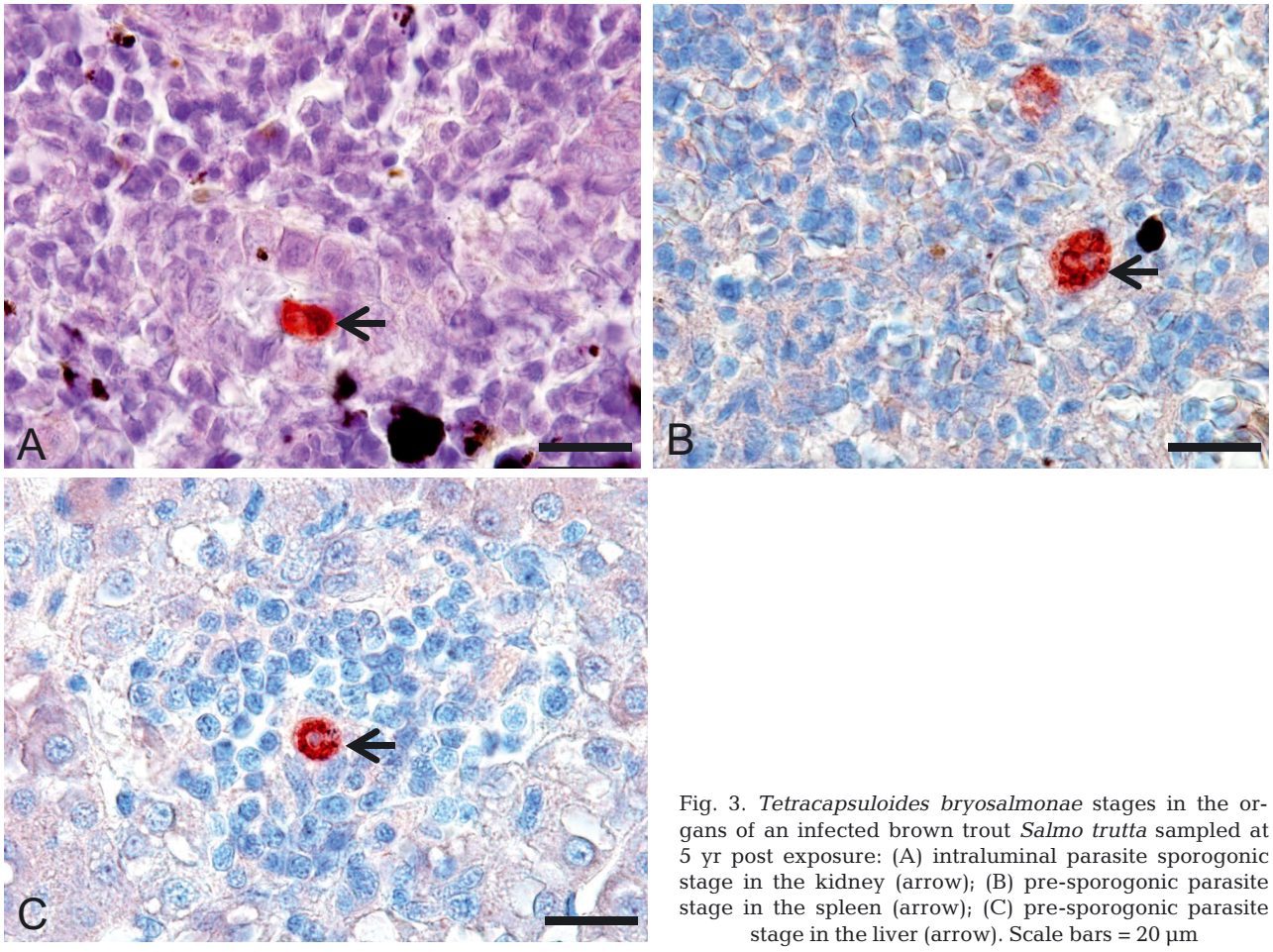

Fig. 3. *Tetracapsuloides bryosalmonae* stages in the organs of an infected brown trout *Salmo trutta* sampled at 5 yr post exposure: (A) intraluminal parasite sporogonic stage in the kidney (arrow); (B) pre-sporogonic parasite stage in the spleen (arrow); (C) pre-sporogonic parasite stage in the liver (arrow). Scale bars = 20 µm

stages were observed in the kidney, spleen and liver of the fish by immunohistochemistry staining which confirmed the persistence of the parasite in the fish. Results from the previous study showed that the clinical signs of PKD disappeared starting from the 52nd week post exposure (Abd-Elfattah et al. 2014b). Similarly, in the present study no lesions or clinical signs were observed in the maintained brown trout 5 yr post infection. Previous reports have attributed the persistence of *T. bryosalmonae* DNA in the fish to either latent infection or residual parasite DNA post infection (Schmidt-Posthaus et al. 2013). In the present study, viable infectious *T. bryosalmonae* (able to cause infection in SPF *F. sultana* colonies) persisted after 5 yr post infection.

Results from the present study indicate that not all *T. bryosalmonae*-infected fish undergo complete clearance of the parasite. Some infected fish become carrier and can disseminate the parasite for prolonged periods of time (at least up to 5 yr). These fish can infect new SPF bryozoan colonies as confirmed by transmission (Fig. 1) and immunohistochemistry (Fig. 3). Spread of the *T. bryosalmonae* through infected statoblasts has been described previously (Abd-Elfattah et al. 2014a, 2017). However, the fact that chronically infected brown trout and bryozoans can release viable spores for several years can explain the wide spread of PKD in open water. It is known that trout are fish from cold water with a physiological optimum temperature reported in the range of 12 to 16°C (Aigo et al. 2014). The brown trout under this study was kept in an aquarium with flow-through system and the mean temperature range was 14 to 16°C in the winter and 16 to 18°C in the summer. This system is a simulation for what happens in the wild. According to our current health monitoring program of brown trout in different Austrian rivers since 2015, the dissemination and prevalence of PKD is very high (authors' unpubl. data), which denotes that recovered brown trout without any clinical signs can release viable spores of *T. bryosalmonae* into the water. Bettage et al. (2009) reported a reduction of *T. bryosalmonae* proliferation in the kidney of infected fish at a water temperature of 12°C. In the same study the authors reported improvement of the parasite development in the kidney by increasing the water temperature to 18°C. Therefore, persistence of *T. bryosalmonae* in recovered brown trout under varying water temperature is most likely.

In conclusion, the results of this study showed that *T. bryosalmonae*-infected brown trout, recovered from the disease, are able to release viable *T. bryosalmonae* for up to 5 yr post infection. This result contributes to explain the high prevalence of *T. bryosalmonae* in the open water (Wahli et al. 2007, Skovgaard & Buchmann 2012, Jenčič et al. 2014, Fontes et al. 2017, Mo & Jørgensen 2017, Vasemägi et al. 2017).

Acknowledgements. This study was funded by the Austrian Science Fund (FWF) project no. P 22770-B17. The study was approved by the institutional ethics committee of the University of Veterinary Medicine, Vienna, and conducted according to Austrian Law for Animal Experiments (Tierversuchsgesetz) 2012, §26, under Permit No. GZ 68.205/0247-II/3b/2011 & GZ 68.205/0141-WF/V/3b/2015

LITERATURE CITED

Abd-Elfattah A, Fontes I, Kumar G, Soliman H, Hartikainen H, Okamura B, El-Matbouli M (2014a) Vertical transmission of *Tetracapsuloides bryosalmonae* (Myxozoa), the causative agent of salmonid proliferative kidney disease. Parasitology 141:482–490

Abd-Elfattah A, Kumar G, Soliman H, El-Matbouli M (2014b) Persistence of *Tetracapsuloides bryosalmonae* (Myxozoa) in chronically infected brown trout *Salmo trutta.* Dis Aquat Org 111:41–49

Abd-Elfattah A, El-Matbouli M, Kumar G (2017) Structural integrity and viability of *Fredericella sultana* statoblasts infected with *Tetracapsuloides bryosalmonae* (Myxozoa) under diverse treatment conditions. Vet Res 48:19

Aigo J, Lattuca ME, Cussac V (2014) Susceptibility of native perca (*Percichthys trucha*) and exotic rainbow trout (*Oncorhynchus mykiss*) to high temperature in Patagonia: different physiological traits and distinctive responses. Hydrobiologia 736:73–82

Bettge K, Wahli T, Segner H, Schmidt-Posthaus H (2009) Proliferative kidney disease in rainbow trout: time- and temperature-related renal pathology and parasite distribution. Dis Aquat Org 83:67–76

Carraro L, Mari L, Hartikainen H, Strepparava N and others (2016) An epidemiological model for proliferative kidney disease in salmonid populations. Parasites Vectors 9:487

El-Matbouli M, Hoffmann RW (2002) Influence of water quality on the outbreak of proliferative kidney disease – field studies and exposure experiments. J Fish Dis 25: 459–467

Feist SW, Bucke D (1993) Proliferative kidney disease in wild salmonids. Fish Res 17:51–58

Feist SW, Peeler EJ, Gardiner R, Smith E, Longshaw M (2002) Proliferative kidney disease and renal myxosporidiosis in juvenile salmonids from rivers in England and Wales. J Fish Dis 25:451–458

Fontes I, Hartikainen H, Holland JW, Secombes CJ, Okamura B (2017) *Tetracapsuloides bryosalmonae* abundance in river water. Dis Aquat Org 124:145–157

Gorgoglione B, Kotob MH, El-Matbouli M (2016) Migrating zooids allow the dispersal of *Fredericella sultana* (Bryozoa) to escape from unfavourable conditions and further spreading of *Tetracapsuloides bryosalmonae.* J Invertebr Pathol 140:97–102

Grabner DS, El-Matbouli M (2008) Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) to

Fredericella sultana (Bryozoa: Phylactolaemata) by various fish species. Dis Aquat Org 79:133–139

Grabner DS, El-Matbouli M (2009) Comparison of the susceptibility of brown trout (*Salmo trutta*) and four rainbow trout (*Oncorhynchus mykiss*) strains to the myxozoan *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease (PKD). Vet Parasitol 165: 200–206

Hedrick RP, MacDonnell E, de Kinkelin P (1993) Proliferative kidney disease of salmonid fish. Annu Rev Fish Dis 3: 277–290

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

Henderson M, Okamura B (2004) The phylogeography of salmonid proliferative kidney disease in Europe and North America. Proc R Soc B 271:1729–1736

Jenčič V, Zajc U, Kušar D, Ocepek M, Pate M (2014) A survey on *Tetracapsuloides bryosalmonae* infections in Slovene fresh waters. J Fish Dis 37:711–717

Kent ML, Khattra J, Hervio DML, Devlin RH (1998) Ribosomal DNA sequence analysis of isolates of the PKX myxosporean and their relationship to members of the genus *Sphaerospora.* J Aquat Anim Health 10:12–21

Kumar G, Abd-Elfattah A, Saleh M, El-Matbouli M (2013a) Fate of *Tetracapsuloides bryosalmonae* (Myxozoa) after infection of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). Dis Aquat Org 107:9–18

Kumar G, Abd-Elfattah A, Soliman H, El-Matbouli M (2013b) Establishment of medium for laboratory cultivation and maintenance of *Fredericella sultana* for *in vivo* experiments with *Tetracapsuloides bryosalmonae* (Myxozoa). J Fish Dis 36:81–88

McGurk C, Morris DJ, Auchinachie NA, Adams A (2006) Development of *Tetracapsuloides bryosalmonae* in bryozoan hosts (as examined by light microscopy) and quantitation of infective dose to rainbow trout (*Oncorhynchus mykiss*). Vet Parasitol 135:249–257

Mo TA, Jørgensen A (2017) A survey of the distribution of the PKD-parasite *Tetracapsuloides bryosalmonae* (Cnidaria: Myxozoa: Malacosporea) in salmonids in Norwegian rivers – additional information gleaned from formerly collected fish. J Fish Dis 40:621–627

Morris DJ, 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

Okamura B, Hartikainen H, Schmidt-Posthaus H, Wahli T (2011) Life cycle complexity, environmental change and the emerging status of salmonid proliferative kidney disease. Freshw Biol 56:735–753

Schmidt-Posthaus H, Steiner P, Müller B, Casanova-Nakayama A (2013) Complex interaction between proliferative kidney disease, water temperature and concurrent nematode infection in brown trout. Dis Aquat Org 104:23–34

Skovgaard A, Buchmann K (2012) *Tetracapsuloides bryosalmonae* and PKD in juvenile wild salmonids in Denmark. Dis Aquat Org 101:33–42

Tops S, Lockwood W, Okamura B (2006) Temperature-driven proliferation of *Tetracapsuloides bryosalmonae* in bryozoan hosts portends salmonid declines. Dis Aquat Org 70:227–236

Vasemägi A, Nousiainen I, Saura A, Vähä J, Valjus J, Huusko A (2017) First record of proliferative kidney disease agent *Tetracapsuloides bryosalmonae* in wild brown trout and European grayling in Finland. Dis Aquat Org 125:73–78

Wahli T, Bernet D, Pascale April Steiner PA, Schmidt-Posthaus H (2007) Geographic distribution of *Tetracapsuloides bryosalmonae* infected fish in Swiss rivers: an update. Aquat Sci 69:3–10

Editorial responsibility: Anindo Choudhury, De Pere, Wisconsin, USA

Submitted: June 15, 2017; Accepted: December 7, 2017
Proofs received from author(s): January 23, 2018