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Development of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) in bryozoan hosts (as examined by light microscopy) and quantitation of infective dose to rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The myxozoan parasite *Tetracapsuloides bryosalmonae* is the causative agent of proliferative kidney disease (PKD), a highly damaging disease of cultured salmonid fish. Within this study, phylactolaemate bryozoans were collected from a river known to be endemic for PKD and subsequently cultured in the laboratory. Sequential developmental stages of *T. bryosalmonae* were studied by light microscopy within the living bryozoan colonies, allowing the identification of stages attached to host peritoneum, consistent with previous molecular evidence of cryptic stages. Infection resulted in the production of large numbers of spores, which were released from the bryozoans. Experimental exposure of rainbow trout (*Oncorhynchus mykiss*) to medium in which infected bryozoans were cultured resulted in clinical PKD. Rainbow trout were exposed to known numbers of *T. bryosalmonae* spores collected by micromanipulation, which had been released from mature spore sacs within colonies of the bryozoan *Fredericella sultana*. Exposure to one spore was sufficient to lead to development of PKD. These findings indicate that small numbers of bryozoans are capable of releasing sufficient spores to infect large numbers of fish, having implications for future control methods for PKD in salmonid farming.
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Keywords: Bryozoa; PKD; PKX; Proliferative kidney disease; Transmission

1. Introduction

Proliferative kidney disease (PKD) is an economically important parasitic condition, primarily affecting first season freshwater salmonid fish in North America and Europe (Hedrick et al., 1993). Seasonal outbreaks of PKD, typically in summer and autumn, have been linked to increased water temperatures which permit severe clinical disease in affected fish (Ferguson and Needham, 1978; Foott and Hedrick, 1987). Clinical disease leads to increased production costs and levels of mortality ranging from below 20% in uncomplicated

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cases, up to 100% in fish suffering from secondary diseases (Ferguson and Ball, 1979; Clifton-Hadley et al., 1986). In the UK alone, losses are estimated at £2.5 M per annum (Feist, 2004), making PKD the most economically significant endoparasitic disease affecting the national trout farming industry.

The causative agent of PKD is the parasite *Tetracapsuloides bryosalmonae*, which was originally known as PKX (Seagrave et al., 1980) before its taxonomic status was resolved (Canning et al., 1999). This parasite and *Buddenbrockia plumatellae* are the only species currently classified within the class Malacosporea of the phylum Myxozoa (Canning et al., 2002). In addition to salmonids, *T. bryosalmonae* infects species of the class Phylactolaemata within the phylum Bryozoa. These bryozoans are a group of ubiquitous freshwater colonial filter-feeding coelomate invertebrates. There have been ultra-structural studies of *T. bryosalmonae* in bryozoans (Canning et al., 1999, 2000) and morphological examination by confocal microscopy of released spores (McGurk et al., 2005). However, these relied upon examination of dissected or fixed material, with inherent limitations due to fixation artefacts, interpretation of plane of section data and the inability to repeatedly inspect individual infected bryozoans. Whereas previous studies have focused on sporogony, the sequential development of *T. bryosalmonae* infection has not been investigated within living bryozoans. Molecular evidence has suggested that other developmental stages exist, which have not been characterised (Tops and Okamura, 2003).

Tetracapsuloides bryosalmonae infection has been successfully induced in rainbow trout (*Oncorhynchus mykiss*) exposed to disrupted infected bryozoan colonies and through cohabitation studies of fish with bryozoans (Feist et al., 2001). It has been suggested that spores of *T. bryosalmonae* are highly effective at locating and infecting trout, with exposure to very low numbers of spores capable of causing PKD (Longshaw et al., 2002). Although quantitation of an infective dose of spores to rainbow trout has been investigated for the myxozoan *Myxobolus cerebralis* (Markiw, 1991), no such information has been gathered previously for *T. bryosalmonae*.

The aims of this study were to observe the sequential development of *T. bryosalmonae* within living bryozoans and determine the minimum number of spores capable of infecting a single fish and inducing disease.

2. Materials and methods

2.1. Collection, laboratory maintenance and examination of bryozoan colonies

Between June 2002 and June 2004, eight field trips were made in the months between February and October to fish farms in southern England known to be endemic for PKD. On each visit, multiple colonies of the bryozoan *Fredericella sultana*, recognised by colony and statoblast appearance (Mundy and Thorpe, 1980), were seen attached to a large willow tree root system within the River Cerne, Dorset (50°47′22″N, 2°28′15″W). Colonies were collected by excising small portions of the root system. Also, colonies of the bryozoan *Plumatella* sp. (identified by colony morphology) adhered to black plastic sheeting were removed from the River Avon, Wiltshire (51°00′09″N, 1°44′41″W) in June 2004. The bryozoans were placed in sealed 5 l plastic boxes filled with aerated river water and transported to the laboratory, where they were attached to 9 cm plastic Petri dishes and cultured at 18 °C in Chalkley's medium (CM: 1.7 mM NaCl, 50 μM KCl, 50 μM $CaCl_2$) following the method of Morris et al. (2002).

The translucent outer cystid of the laboratory-cultured bryozoans allowed daily observation for myxozoan parasitism using a dissecting microscope (SZ30, Olympus, Tokyo, Japan) and an inverted microscope (CK2, Olympus). Details of the timing between collection and first evidence of infection were noted as were developmental features of the parasite within the host. A 35 mm camera (SC35, Olympus) was used for still images, while a video camera (F15, Panasonic, Tokyo, Japan) was used for moving image capture. Video capture software (Version 7.5, ATI, Markham, Canada) was used to digitise the images.

2.2. Experimental challenge of fish with culture media from infected colonies of F. sultana

Twenty-four rainbow trout, mean weight 85 g, from a PKD-free source were randomly divided into two groups in 100 l flow-through tanks supplied with

dechlorinated tap water at 18 °C in the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling. After acclimatisation, the water flow was stopped to both tanks and the level reduced to 21 l with continuous, vigorous aeration applied. To one of the tanks, 1 l of CM was added from a tank containing parasitised colonies of *F. sultana*. To the other tank, acting as a negative control, freshly made CM was added. After 60 min, the water supplies to both tanks were restarted. The exposure procedure was repeated after 4 days.

After 50 days maintenance at a water temperature of 18 °C, the fish were overdosed with 10 mg l^{-1} benzocaine and killed by severing the spinal cord. Samples of kidney were fixed in 10% neutral buffered formalin for 24 h for examination by immunohistochemistry. Samples of kidney were also taken from each fish and frozen at −20 °C for subsequent polymerase chain reaction (PCR) analysis.

2.3. *Quantitation of the minimum infective dose of T. bryosalmonae spores to rainbow trout*

2.3.1. *Spore collection*

Colonies of *F. sultana*, collected and maintained as described above, were examined regularly until identifiable spores of *T. bryosalmonae* appeared within the metacoel. The bryozoans were teased apart using scalpel blades to release the spores into the surrounding medium. An IM-6 microinjector (Narishige, Tokyo, Japan) was connected to an oil-hydraulic MO-155 micromanipulator (Narishige) attached to an Olympus LH50A inverted microscope stage. Stretched glass capillary tubes (GD-1, Narishige) were attached to the microinjector for spore collection.

2.3.2. *Exposure trials*

Two exposure trials with *T. bryosalmonae* spores administered to rainbow trout were conducted. For the first trial, the spores were counted, as they were drawn into the micropipette. Known numbers of spores from each withdrawal were then expelled into individual bijoux. To each of 6 replicate containers, 1 spore was added, while 6 replicate containers were also made with 5 and 10 spores. For negative controls, six bijoux containing only freshly made CM were used.

Twenty-four rainbow trout (mean weight 16 g) originating from a PKD-free source were each allocated to one of 24, 10 l flow-through tanks maintained at 18 °C in the ARF. Immediately prior to challenge, the water supply to each tank was stopped and the volume of each tank reduced to 2.5 l. One of the containers described above was randomly assigned to each tank. The content of each container was added to its designated tank, with thorough rinsing of the bijou with tank water. The tanks were maintained with vigorous aeration at 2.5 l for 1 h, after which the water supply to each tank was restarted.

For the second trial, the spores were not counted as they were drawn into the micropipette but rather as they were slowly expelled onto individual cavity microscope slides, while being viewed using the inverted microscope. Between 1 and 16 spores were added to each slide. These slides were kept cool on ice until being transported within 15 min to the ARF.

Twenty-four rainbow trout (mean weight 25 g) from a PKD-free source were allocated as described above. Again, prior to exposure, the water flow was halted and the level reduced to 2.5 l and to each tank, one cavity slide was randomly assigned, the slide being well rinsed in the tank water and then attached to metal clips and suspended vertically by string in the tank for 1 h. To each of six negative control tanks, a slide including freshly made CM was added.

The fish from the first and second trials were maintained at 18 °C for 56 and 63 days, respectively, after which they were killed and sampled as described above.

2.3.3. *Examination of tissue sections by immunohistochemistry*

Fixed kidney samples from the above trials were paraffin-embedded, sectioned at 5 μm and examined by immunohistochemistry using the *T. bryosalmonae* specific monoclonal antibody P01 (Aquatic Diagnostics Ltd., Stirling, UK) following the manufacturer's instructions.

2.3.4. *DNA amplification and detection*

Kidney samples from the trials detailed above were defrosted and transferred to sterile 1.5 ml Eppendorf tubes. A DNA extraction kit (Genisol Maxi-Prep Kit, ABgene, Epsom, UK) based on the protocol of Miller et al. (1988) was used for DNA extraction.

The *T. bryosalmonae* specific primers 5F and 6R designed by Kent et al. (1998) were used for

amplification of parasite DNA extracted from the kidney samples. The PCR was conducted using a mastermix solution (Reddymix, ABgene), with the cycling parameters recommended by Kent et al. (1998). For each reaction, positive control samples including DNA extracted from a PKD-affected rainbow trout kidney and negative control samples including ultra-pure water instead of DNA template were included alongside the samples being analysed. The PCR products were visualised following electrophoresis on a 1.5% agarose gel.

3. Results

3.1. The development of *T. bryosalmonae* within bryozoans

3.1.1. Recognition of infection

From each sampling trip, malacosporean development was seen within the bryozoans. The time interval between collection and identification of parasite development varied from 2 to 38 days with a mean of 22 days. Following initial recognition of infection, tetracapsulid spores were observed within 5 days. The identity of the parasite was confirmed as *T. bryosalmonae* by the development of PKD in fish exposed to the spores as described below.

3.1.2. Developmental stages of *T. bryosalmonae* within phylactolaemate bryozoans

Bryozoan proliferation, including the production of spermatozoa, was seen to consistently precede observed malacosporean development (Fig. 1a). The initial indicator of *T. bryosalmonae* infection was the presence of numerous particles of approximately 7 μm diameter swirling within the bryozoan metacoel. Within a day, spheroid stages of 8–15 μm diameter were observed attached to the bryozoan peritoneum (Fig. 1b), being particularly evident near the retractor muscles of the lophophore.

After 1–3 days, irregularly shaped bodies of 40–100 μm diameter were circulating within the metacoel (Fig. 1c). These varied in shape from spheroid to ellipsoid (Fig. 1d), with irregular protuberances on their external surfaces. During days 4–5, spherical sac-like bodies of 50–100 μm diameter became evident within the metacoel. Initially, these sacs appeared empty (Fig. 1e), with subsequent development of refractive bodies that were located peripherally within the sacs, giving the appearance of a central lucent area surrounded by a refractive layer (Fig. 1f). Later, the refractive bodies coalesced, filling the lumina of the sacs (Fig. 2a), with abundant spherical sacs of *T. bryosalmonae* seen within single zooids (Fig. 2b). Protuberances could be seen from the external walls of some otherwise regularly spherical sacs that were slowly moving within coelomic currents (Fig. 2c). The protuberances were of 15–50 μm diameter, ranging from simple to complex in structure (Fig. 2d).

Upon maturity, spherical spore sacs of diameter 200–350 μm were packed with refractive material. Within 1 or 2 days of this appearance, spore sacs ruptured releasing many spores that swirled within the metacoel alongside other intact spore sacs and immature *T. bryosalmonae* stages. It was estimated that there were many thousands of spores within one small bryozoan colony composed of just five zooids. Their fast movement within the bryozoan host made examination difficult, although identification could be discerned where spores became located in slow moving coelomic currents (Fig. 2e). Following the observation of spores within individual colonies, their prevalence became markedly diminished within 12 h, with no spores identifiable after 24 h.

Examination of dissected material using an inverted microscope confirmed the general structure of the spore sacs, with recognisable spores seen exiting mature sacs following dissection. Each spore approximately 20 μm diameter included four spherical polar capsules and two germinative sporoplasms (Fig. 2f). Although some infected bryozoan colonies died following infection, this was not universal. Some colonies showed no sign of overt infection following the release and dissipation of spores. However, following successful maintenance, signs of infection returned within 10–20 days. Repeated waves of infection were observed, with both immature and mature parasitic stages being synchronously present within individual colonies.

3.2. Challenge of fish with culture media from infected colonies of *F. sultana*

All of the 12 fish exposed to medium containing infected bryozoans showed nephromegaly of grades

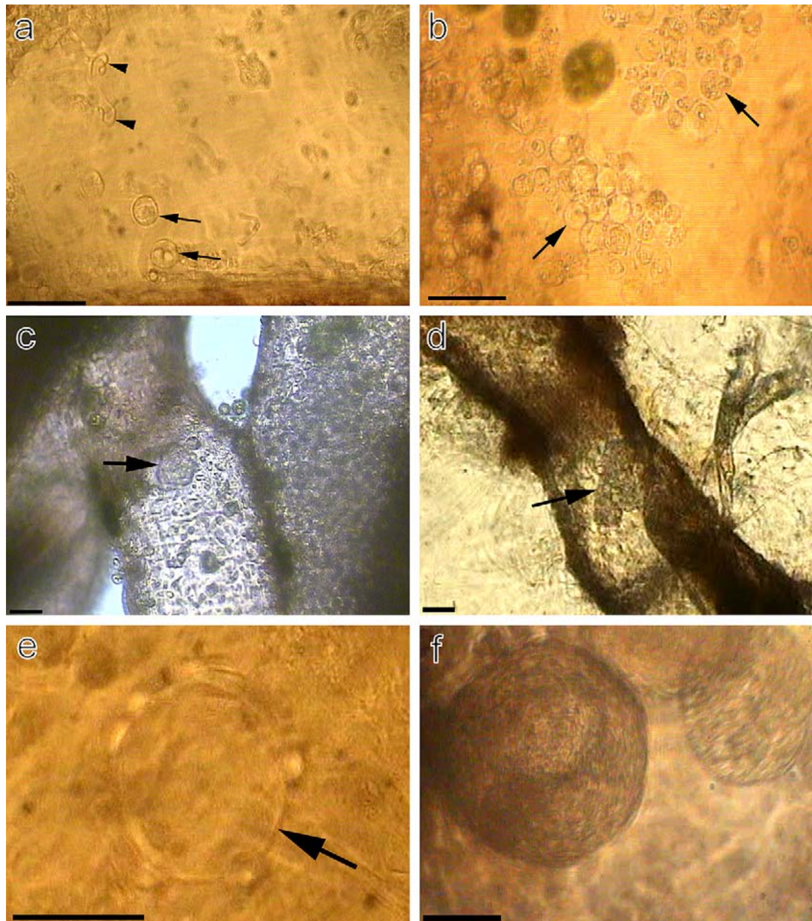

Fig. 1. Light micrographs of *T. bryosalmonae* within *F. sultana* (a–e) and *Plumatella* sp. (f), all bars = 50 μm. (a) Bryozoan spermatozoa (arrowheads) and *T. bryosalmonae* spores (arrows) can be seen. (b) Round parasitic stages (arrows) are attached to the host peritoneum. (c) Small irregular freely circulating parasite (arrow). The zooid on the right is packed with released spores. (d) A more mature irregular parasitic stage (arrow). (e) An early developmental spore sac (arrow) with empty lumen and external surface protuberances. (f) A lucent central zone is present within the developing spore sac on the left.

1–2 according to the scale of Clifton-Hadley et al. (1987), with the presence of *T. bryosalmonae* confirmed by immunohistochemistry and PCR. None of the negative control fish tested positive.

3.3. Quantitation of the minimum infective dose of *T. bryosalmonae* spores to rainbow trout

In the first trial, only one fish, which had been exposed to a single spore was infected. This fish exhibited characteristic signs of PKD with numerous interstitial cells of *T. bryosalmonae* identifiable (Clifton-Hadley et al., 1987). In the second trial, five fish exposed to 2, 5, 6, 14 or 16 spores were infected. There was no evidence of a relationship between exposure dose and pathological response, with fish exposed to one or six spores developing severe interstitial nephritis, while those exposed to 14 or 16 spores showed only mild signs. None of the negative control fish tested positive.

4. Discussion

The collection of wild bryozoan colonies with subsequent laboratory maintenance and microscopical

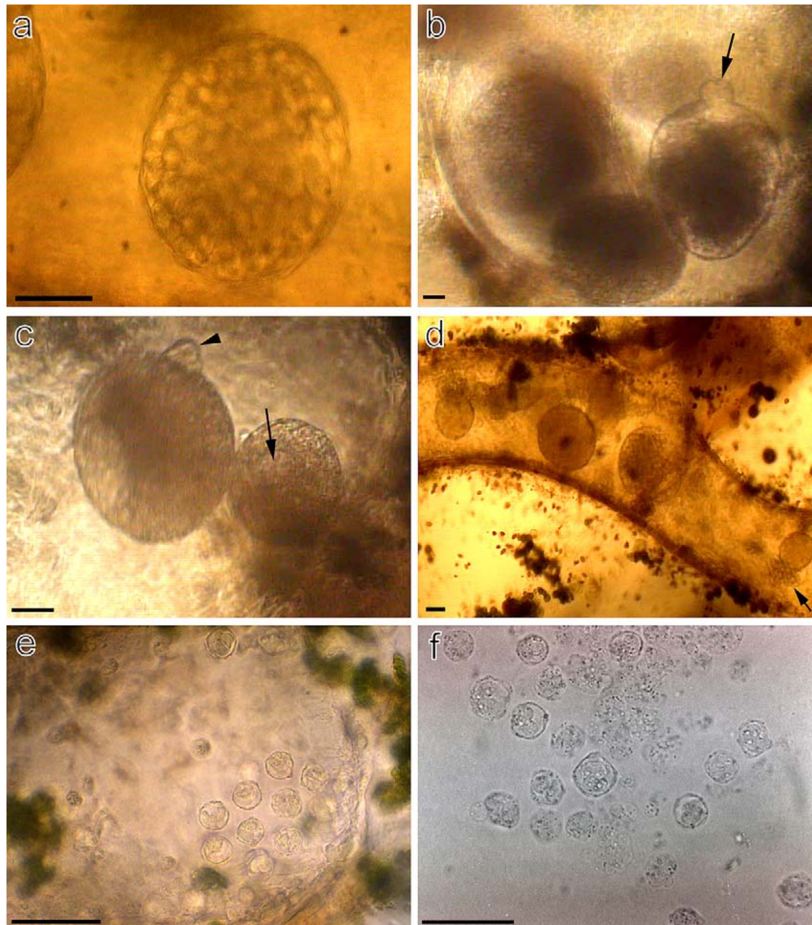

Fig. 2. Light micrographs of *T. bryosalmonae* within *F. sultana* (a, d and e) and *Plumatella* sp. (b and c), all bars = 50 μm. (a) A maturing spore sac containing refractive developing spores. (b) Protuberances (arrow) can be seen from the surface of some developing sacs. (c) Developing spore sacs exhibiting both protuberances (arrowhead) and central lucent zones (arrow) can be seen concurrently within bryozoans. (d) Many spore sacs could be seen within single colonies, one of which exhibits irregular surface protuberances (arrow). (e) Mature spores released into the bryozoan metacoel. (f) Dissected spores, displaying twin sporoplasms and four spherical polar capsules.

observation has allowed the first documented study of the sequential development of *T. bryosalmonae* within living invertebrate hosts. The suggestion that cryptic stages of *T. bryosalmonae* exist within bryozoan tissue, subsequently leading to observed infection (Tops and Okamura, 2003) is consistent with our finding that a delay occurred between collection of specimens and recognition of infection. The first sign of overt *T. bryosalmonae* infection was the presence of numerous small swirling particles, although it could not be ascertained if they were parasitic cells or coelomic corpuscles resulting from host response to infection (Mano, 1964). Subsequently, spheroid stages of *T. bryosalmonae* were adhered to the bryozoan peritoneal wall, followed by freely circulating coelomic bodies. The observation of the early stages of *T. bryosalmonae* attached to host tissue identifies previously elusive stages and suggests that further developmental stages are present within the body wall itself.

The earliest spore sacs of *T. bryosalmonae* previously observed possessed cavities already packed with cells (Canning and Okamura, 2004). Our light microscopical study has shown that proliferative cells originate from the wall of the sac before coalescing to fill the lumen, concurring with previous descriptions of *B. plumatellae* infection (Canning et al., 2002) and an

unidentified (putatively malacosporean) parasite of the bryozoan *Lophopus crystallinus* (Allman, 1856). In *T. bryosalmonae*, further differentiation of these proliferative cells results in populations of stellate and sporoplasmogenic cells for subsequent sporogony (Canning et al., 2000). Immature *T. bryosalmonae* spore sacs are not regularly spherical, but can feature simple or elaborate external protuberances that are subsequently lost, suggesting that they are either resorbed or bud to form new sacs as proposed for similar structures observed during a light microscopical examination of *B. plumatellae* infection (Okamura, 1996).

Upon maturation of the spore sacs of *T. bryosalmonae*, large numbers of spores were released within the host metacoel, as also reported for a *Buddenbrockia* sp. (Morris et al., 2002). The dissipation of spores within 24 h of their release suggested that they were subsequently discharged into the surrounding culture medium presumably through the bryozoan vestibular pore, as proposed with *Buddenbrockia* sp. (Morris et al., 2002). Despite extensive monitoring, no intact mature spore sacs were witnessed leaving the bryozoans within the current studies, in contrast to observation of *B. plumatellae* (Canning et al., 2002). The release of mature *T. bryosalmonae* spores into the external environment was exploited in the experimental exposure of rainbow trout to the bryozoan culture medium. Fish were successfully infected following two exposures of 60 min duration 4 days apart. As the water volume was not reduced excessively during the exposure, it could be interpreted that *T. bryosalmonae* is proficient at contacting fish tissue as previously suggested by Feist et al. (2001). These authors transmitted *T. bryosalmonae* infection by exposing trout for 90 min to disrupted infected bryozoan material and by long-term cohabitation with infected bryozoans, but not by short-term exposure to culture medium alone. Similarly, Longshaw et al. (2002) successfully infected rainbow trout following exposure to homogenised bryozoans. The current study demonstrates that infection can be established in fish without direct contact with bryozoans. Thus, this procedure is non-destructive for the alternate host of the parasite, allowing preservation of valuable infected colonies.

It has previously been suggested that a low dose of spores might be able to elicit infection in fish, although no quantitative data were available (Feist et al., 2001; Longshaw et al., 2002; Tops et al., 2004). In our first quantitation trial, a fish exposed to just one spore became infected, although those exposed to more spores did not. This could be attributed to degradation following dissection, as *T. bryosalmonae* spores are considered to be relatively labile (de Kinkelin et al., 2002). The cooling of samples and reduced handling time during the second trial resulted in the infection of five fish suggesting that the experimental was still not optimal. The reason for this is not clear, however, the protocol could have caused acute stress to the fish, leading to increased mucous cell discharge into the reduced water volume (Nolan et al., 2000). Exposure to mucus has been associated with premature firing of myxozoan polar filaments (Xiao and Desser, 2000), potentially contributing to the low incidence of experimental *T. bryosalmonae* infection. Gay et al. (2001) suggested that the severity of the immune response in trout kidney to extrasporogonic stages of *T. bryosalmonae* was more dependent upon the amount of parasite encountered than the ambient temperature. However, the absence of a relationship between spore challenge quantity and resultant pathological severity in our trials contrasts with the linear dose response seen in rainbow trout exposed to the triactinomyxon spores of the myxozoan *M. cerebralis* (Markiw, 1992). It has been suggested that the developmental course of PKD in individual fish depends upon a combination of genetic and environmental factors (Morris et al., in press). Therefore, the dose of *T. bryosalmonae* spores necessary to elicit infection and subsequent rate of development of PKD may vary between individuals within a population of fish. Future trials should examine wider differentials in spore challenge numbers and resultant pathological signs.

Canning et al. (2000) suggested that upon entry of *T. bryosalmonae* stages into fish tissue, two haploid secondary cells released from the sporoplasms of the spore would fuse together resulting in fertilisation. It was speculated that due to the relatively small number of spores synchronously contacting fish, the process would result in autogamy, with two secondary cells from a single spore fusing. The current finding of development of infection in fish exposed to only one spore supports this hypothesis. As the pathological appearance of *T. bryosalmonae* infection from such low spore numbers did not differ from established descriptions (Clifton-Hadley et al., 1987), it would

seem likely that, at least some, if not all clinical cases of PKD result from autogamous parasite reproduction.

It had previously been suggested that reducing potential bryozoan habitats, such as submerged vegetation and wood debris for several hundred metres upstream of the inlets to PKD-endemic farms could act as an effective control measure against PKD (de Kinkelin et al., 2002). However, bryozoan colonies can be difficult to find, with *T. bryosalmonae* infection having been found in an experimental recirculation system although no infected colonies were identified despite extensive scrutiny (de Kinkelin et al., 2002). The present study has demonstrated that large numbers of highly infective *T. bryosalmonae* spores are recurrently produced and released from relatively small, infected bryozoan colonies. This explains the high morbidity levels experienced on affected farms (Seagrave et al., 1981). Difficulties in being able to entirely eradicate bryozoan populations from inlet screens, filters and pipe-work could prove a major obstacle to controlling PKD on farms, emphasising the importance of investigating alternative control measures including vaccination, chemotherapy and husbandry manipulation.

5. Conclusions

The maintenance and study of living bryozoan colonies infected with *T. bryosalmonae* allowed the recognition of previously undescribed phases of parasitism. Sequential waves of parasite development mean that infection can be maintained for prolonged periods within laboratory systems. The development of an experimental fish challenge method, which was non-invasive to bryozoans could be valuable in future investigations of host–parasite interactions. Observation of the massive production and release of *T. bryosalmonae* spores from bryozoans, in conjunction with quantitation of the infectious dose of spores to rainbow trout has implications for understanding the epidemiology of PKD and for future development of control strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2005.07.022.

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