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Infection of bryozoans by *Tetracapsuloides bryosalmonae* at sites endemic for salmonid proliferative kidney disease

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ABSTRACT: Laboratory-reared colonies of the bryozoans *Fredericella sultana* and *Plumatella fungosa* were placed upstream of 2 fish farms endemic for salmonid proliferative kidney disease (PKD) to assess rates of infection of bryozoans by *Tetracapsuloides bryosalmonae*, the causative agent of PKD. Colonies were deployed in the field for 8 trial periods of 2 wk each throughout the summer of 2001. Following each trial, bryozoan colonies were maintained in laboratory culture for 28 d and were regularly monitored for infection by searching for sac stages of *T. bryosalmonae*. Infections were never identified by observations of sac stages, however positive PCR results and sequencing of cultured material confirmed that cryptic infections were present in colonies of both species deployed at one site. The possibility that PCR results reflected contamination of surfaces of bryozoans can be excluded, given the short period of spore viability of *T. bryosalmonae*. Highest rates of infection occurred when 4 of 23 colonies of *F. sultana* and 1 of 12 colonies of *P. fungosa* were infected during the period 10 to 24 July. No infections were detected from mid-August to late October at this site. None of the colonies at the other site became infected throughout the period of study. Our data provide the first estimates of infection rates of bryozoans by *T. bryosalmonae*. Additionally, they provide evidence that a cryptic stage can be maintained within bryozoan hosts for a period of 4 to 6 wk.

KEY WORDS: *Tetracapsuloides bryosalmonae* · Proliferative kidney disease · Freshwater bryozoans · Infection rates · Cryptic stages · Malacosporea · Myxozoa

INTRODUCTION

Proliferative kidney disease (PKD) is a seasonal disease affecting wild and cultured salmonid fish, and is caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* (formerly *Tetracapsula bryosalmonae*: Canning et al. 1999, 2002). Analysis of 18S rDNA sequences revealed that *T. bryosalmonae* develops in freshwater bryozoans (phylum Bryozoa; class Phylactolaemata) (Anderson et al. 1999a), and subsequent transmission studies established that infective spores released from bryozoans produce PKD in fish (Feist et al. 2001). PKD is manifested as a massive immune response to the presence of *T. bryosalmonae*, and peaks in summer as a result of the temperature-dependency of the fish immune system (Manning & Nakanishi 1996). The disease poses significant economic threat due to stock losses suffered in the salmonid farming and hatchery industries in Europe and North America (Hedrick et al. 1993).

The phylum Myxozoa contains some 1350 species, which occur as endoparasites in fish and invertebrates (Kent et al. 2001). Most species have been described from fish, and the small number of life cycles that have been resolved incorporate both fish and invertebrate hosts. The majority of species within the phylum Myxozoa belong to the class Myxosporea. *Tetracapsuloides bryosalmonae* is one of only 2 species described in the class Malacosporea. Both malacosporeans parasitise freshwater bryozoans (Canning et al. 2000) and, unlike myxosporeans, produce soft, unprotected spores with a limited period of viability. Many aspects of the biology of malacosporeans remain obscure.

Freshwater bryozoans are sessile, colonial invertebrates that are common in both lotic and lentic habitats. They undergo prolific colony growth in summer

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and produce dormant, seed-like propagules (statoblasts) which overwinter and hatch into small colonies when favourable conditions return. Some species also overwinter as colonies (Raddum & Johnsen 1983). Mature stages of *Tetracapsuloides bryosalmonae* occur as free sacs containing infective spores in the coelomic cavity of bryozoan hosts (Canning et al. 1999, 2000). The sacs are entrained with the general circulation of the host's coelomic fluid and can be observed within bryozoan colonies with the dissection microscope. So far, 5 bryozoan species have been identified as hosts of *T. bryosalmonae* (Anderson et al. 1999b, Longshaw et al. 1999, Okamura et al. 2001), but knowledge of the ecology and complete life cycle of *T. bryosalmonae* is limited.

We undertook this study to enhance our understanding of the ecology and life cycle of *Tetracapsuloides bryosalmonae* by assessing spatial and temporal variation in infection rates of bryozoans and the development of infections within bryozoan hosts.

MATERIALS AND METHODS

The phylactolaemate bryozoans *Fredericella sultana* and *Plumatella fungosa* were cultured in laboratory aquaria in constant conditions of temperature (20°C) and light using the method of Wood (1971). The *F. sultana* material was obtained from ongoing culture of colonies originating from collections in the River Kennet, Berkshire, UK, in 2000. The *P. fungosa* material had been maintained in long-term culture**,** with generations of colonies being derived from statoblasts. The original source of this *P. fungosa* material was a colony collected in July 2000 from Blenheim Lake, Oxfordshire, UK. *Tetracapsuloides bryosalmonae* infections were never observed during routine observations of cultured *F. sultana* or *P. fungosa.* In addition, our stock of uninfected cultured material was maintained in separate culture systems from those to which colonies were returned after field trials (see below) and so was never in contact with potential infections. The material was therefore considered to be parasite-free, but this assumption was further checked by undertaking polymerase chain reaction (PCR) (see below) of portions of the colonies used as source material for field trials. None of the PCR results provided evidence of infection.

Branches from colonies of each species were induced to attach by laying them on plastic discs (diameter = 5.4 cm) in petri dishes with water from the culture system. Once branches had attached to the discs, they were moved to the culture system for onward colony growth. Colonies on the discs which contained >10 zooids were randomly slotted into positions on a Perspex rod, and this assembly was then placed inside plastic cages (bird-feeders). Cable ties were used to close the cages around the discs. Each cage contained 4 *Fredericella sultana* and 2 *Plumatella fungosa* colonies. Cages were placed upstream of fish farms endemic for PKD by suspending the cages with lead weights into the mainstream channel flow at 2 sites: 1 site was a channel diverted from the River Kennet and leading into a rainbow trout farm in Berkshire, the other was a channel diverted from the River Itchen into a rainbow trout farm in Hampshire. The cages remained in the field for trial periods of 2 wk each, when they were then replaced with a new set of bryozoans in cages; towards the end of the study, cages remained in the field for 4 wk periods. A total of 6 cages were deployed at each site, giving a total of 24 colonies of *F. sultana* and 12 colonies of *P. fungosa* per trial with 2 exceptions: 18 colonies of *F. sultana* were used in Trials 4 and 6 in the channel from the River Kennet. The bryozoans which survived exposure to field conditions were returned to laboratory culture systems for 28 d. During this culturing period they were inspected for infections of *Tetracapsuloides bryosalmonae* by searching for the presence of sacs in the body cavity of bryozoans every 2 to 5 d using a dissection microscope. The 28 d period of laboratory culturing was based on the time it takes for PKD to develop in fish (Hedrick et al. 1993) and the capacity to accommodate colonies from several trial periods simultaneously in the culture system. This period greatly exceeds spore viability in *T. bryosalmonae*, which is lost between 12 and 24 h (de Kinkelin et al. 2002). Colonies surviving the 28 d culture period were preserved in 99% ethanol at –20°C for PCR of individual colonies, with the exception of colonies from Trial 1 which were pooled by species for each cage. In all cases, colonies that were saved for PCR had not been in contact with field-collected material for at least 7 d. Given these protocols and spore viability, it is highly unlikely that extraneous *T. bryosalmonae* spores associated superficially with colony surfaces would have contaminated colonies preserved for PCR.

DNA extraction was carried out using a standard CTAB hexadecyltrimethylammonium bromide-proteinase-K method, as described by Winnepenninckx et al. (1993), with the following modifications: (1) samples were stored at –20°C, (2) the samples were not ground in liquid nitrogen prior to extraction, (3) samples were suspended in 675 µl preheated (60°C) CTAB buffer, (4) all steps were carried out using 1.5 ml plastic Eppendorf tubes. PCR was conducted using *Tetracapsuloides bryosalmonae* specific primers 514F and 776R with an internal competitive standard mimic molecule developed by Morris et al. (2002). The inclusion of an internal competitive standard allows the verification of PCR

results by identifying false negative results. This procedure was necessary due to inherent problems with inhibition when conducting PCR on DNA derived from some freshwater bryozoans. Each set of amplifications further included positive and negative controls. Products (262 bp) from 3 randomly chosen positive PCR samples were inserted into T-Easy vector (Promega) and 1 clone of each was sequenced using a standard sequencing reaction on an ABI 3100 automated sequencer.

RESULTS

Colony mortality occurred in both the field and during ongoing culturing. The total numbers of colonies at the end of each study period were therefore not equivalent to the numbers at the start (Table 1). There was a trend towards increased mortality in both the field and subsequent laboratory culturing for *Plumatella fungosa* over the course of the study. In contrast, with some exceptions, *Fredericella sultana* showed reasonably high survivorship throughout the study. Both species underwent notable mortality during culture of Trial 7 material from both sites.

Visual inspection of colonies during culturing revealed no signs of infection by *Tetracapsuloides bryosalmonae*. However, PCR revealed that *T. bryosalmonae* was present in some colonies deployed in the channel from the River Itchen. At this site, highest infection rates were detected during Trial 2, when 4 *Fredericella sultana* colonies and 1 *Plumatella fungosa* colony were infected (Table 2). Due to pooling of colonies of the same species within cages in Trial 1, the actual number of colonies infected could not be determined. We report the inferred minimum number of infected *F. sultana* colonies from Trial 1 as 2 out of 18 colonies (pooled *F. sultana* colonies from 2 of 5 cages provided positive PCR results); 1 *F. sultana* colony became infected in Trial 4. There was no significant difference between the number of infected and uninfected colonies of *F. sultana* and *P. fungosa* ($\chi^2 = 1.979$, df = 1, $p > 0.05$: analysis of data collected through Trial 4 since no infections were detected after this period). None of the colonies deployed in the channel of the River Kennet ever became infected (Table 2).

Of the 3 sequences of clones obtained from the 3 randomly chosen positive PCR results, 2 were identical to a *Tetracapsuloides bryosalmonae* sequence in GenBank (AF190668), which originated from an infected *Plumatella emarginata* colony from the River Cerne in southern England (Longshaw et al. 1999). The third sequence differed by 1 bp, which could be attributable to sequencing error.

Table 1. *Fredericella sultana* and *Plumatella fungosa*. Numbers of colonies deployed (Dep) at fish farms over 8 trial periods (2 wk), numbers returned alive to laboratory (Rtn) and numbers which survived 28 d culture period and were used for PCR analysis (PCR). nd: no colonies deployed

Trial	Date deployed (d/mo/yr)	*F. sultana* Dep	*F. sultana* Rtn	*F. sultana* PCR	*P. fungosa* Dep	*P. fungosa* Rtn	*P. fungosa* PCR
River Itchen							
1	26/06/01	24	23	18	12	11	10
2	10/07/01	24	23	23	12	12	12
3	24/07/01	24	22	23	12	9	7
4	07/08/01	24	24	22	12	12	12
5	21/08/01	24	12	11	12	5	4
6	04/09/01	24	22	19	12	11	3
7[a]	18/09/01	24	24	12	12	7	0
8[a]	16/10/01	24	24	20		nd	
River Kennet							
1	19/06/01	24	20	20	12	6	6
2	03/07/01	24	23	23	12	11	10
3	17/07/01	24	22	19	12	10	6
4	31/07/01	18	13	12	12	7	7
5	14/08/01	24	23	20	12	7	6
6	28/09/01	18	13	11	12	8	6
7	12/09/01	24	24	14	12	12	0
8[a]	25/09/01	24	8	5	12	0	0

[a]Colonies in field for 4 wk

DISCUSSION

Infection rates

Our study has shown that rates of infection of bryozoans by *Tetracapsuloides bryosalmonae* can be investigated by placing uninfected bryozoan colonies into environments enzootic for PKD. Both *Fredericella sultana* and *Plumatella fungosa* became infected during 2 wk trial periods in the channel from the River Itchen. The highest rate of infection was detected during early to mid-July, when 4 out of 23 colonies (~17%) of *F. sultana* and 1 out of 11 colonies (~9%) of *P. fungosa* were infected during the 2 wk period. The first infections were picked up in late June/early July, and 1 *F. sultana* colony became infected during early to mid-August. The limited viability of *T. bryosalmonae* spores (less that 24 h) (de Kinkelin et al. 2002) means that our positive PCR results cannot be explained by contamination of material by spores adherent to external surfaces

Table 2. *Fredericella sultana* and *Plumatella fungosa*. Number of infected colonies (Inf), and number of colonies surviving field deployment and subsequent laboratory culturing for 28 d (Surv); na: not available. See Table 1 for trial dates

Trial	*F. sultana*		*P. fungosa*	
	Inf	Surv	Inf	Surv
River Itchen				
1	2	18[a]	0	10
2	4	23	1	12
3	0	23	0	7
4	1	22	0	12
5	0	11	0	4
6	0	19	0	3
7	0	12	na[b]	
8	0	20	na[c]	
River Kennet				
1	0	20	0	6
2	0	23	0	10
3	0	19	0	6
4	0	12	0	7
5	0	20	0	6
6	0	11	0	6
7	0	14	na[b]	
8	0	5	na[d]	

[a]Minimum number infected due to pooling of material (see 'Results')
[b]All colonies died in culture prior to PCR analysis
[c]No colonies deployed
[d]All colonies died prior to return from the field

of the bryozoans, since the bryozoans were subsequently maintained in culture for 28 d prior to fixation for PCR. No infection was observed in material deployed from mid-August to late-October. The peak in prevalence of PKD in rainbow trout *Oncorhynchus mykiss* in the fish farm directly downstream of the trial site occurred in August (N. McFarling pers. comm.). Laboratory transmission studies (Feist et al. 2001) suggested that this peak in PKD prevalence resulted from infection some 4 wk earlier, coinciding with the period of peak infection rates of bryozoans.

None of the 207 colonies which survived field deployment in the channel from the River Kennet and subsequent laboratory culture were infected during the 8 trials, although the fish on the farm downstream developed PKD during the period of study (S. Leach, pers. comm.). The lack of infections in the River Kennet material is highly unlikely to be due to lack of detection by PCR, since we included the internal competitive standard and positive and negative controls with every amplification. Indeed, the consistent negative results for this trial site are suggestive of some other explanation.

The absence of infection in bryozoans deployed in the channel from the River Kennet could simply reflect a sampling effect resulting from the relatively small number of colonies used during each trial, or low concentrations of infective spores at the site. Another explanation could be that the colonies may not have been exposed to infective spores. This could occur if spores were concentrated in different regions of the water column from those sampled for caged bryozoans. Another possibility is that the dense aggregations of suspension-feeding simuliid larvae which developed on cages at this site may have reduced spore supply to the bryozoans. Simuliids were rare at the other site (S. Tops unpubl. data). Alternatively, the bryozoan material may have been resistant to the particular strain of *Tetracapsuloides bryosalmonae* in the River Kennet. Recent research has revealed differential infection of host genotypes of *Tubifex tubifex* by the myxozoan *Myxobolus cerebralis* (El-Matbouli et al. 1999, Beauchamp et al 2001, 2002). The *Fredericella sultana* colonies were derived from stock which originated from the site, and it is possible that this source population has developed resistance to the local strain of *T. bryosalmonae*. Failure to detect infection by PCR in colonies of *F. sultana* collected regularly from the site would support this view (S. Tops & B. Okamura unpubl. data), and implies that infected bryozoans further upstream represent the source of PKD infection to fish. On the basis of this argument, the *Plumatella fungosa* colonies might be expected to have been susceptible to infection since they were derived from material collected from another site. On the other hand, only 1 *P. fungosa* colony became infected at the other site. It is evident that estimation of infection rates of both bryozoan species, but particularly of *P. fungosa*, would benefit from larger sample sizes.

Despite the problems imposed by low sample sizes and various other factors that could have compromised exposure of experimental colonies to infective spores, the peak rate of infection suggests that at certain times there is a substantial capacity for infecting bryozoan populations in the River Itchen system. Since *Fredericella sultana* is capable of forming dense stands of large, intertwining colonies, an infection rate of 17% (4 out of 23) of small colonies over a 2 wk period could translate into the development of a large number of infected colonies in local bryozoan populations, leading to the eventual release of enormous numbers of infective spores. The high prevalence of PKD at the site during the summer of 2001 and in previous years (up to 100%; N. McFarling pers. comm.) is a reflection of the large numbers of infective spores that are regularly present.

It should be noted that our estimates of rates of infection are minimal, since it could not be determined whether colonies which died during the course of the study in the field and in culture were infected. DNA degradation as a result of mortality precluded PCR testing of dead bryozoans for infection. Undetected infec-

tion may well have contributed to such mortality. However, even if the true infection rates were higher than those estimated by our approaches, the infection rates of practical interest are those that will result in the development of parasites through to mature spore-producing sacs in their bryozoan hosts. The development of *Buddenbrockia plumatellae* — the closest known relative of *Tetracapsuloides bryosalmonae* (Canning et al. 2002) — in less than 5 d suggests that we could have missed infections on the rare occasions when there was a 5 d gap between colony observations. This seems unlikely, however, since infected colonies remain alive for at least several days (authors' pers. obs.).

Development of cryptic stages in bryozoan hosts

Our study provides evidence that *Tetracapsuloides bryosalmonae* persists as cryptic stages in bryozoan hosts. We failed to detect stages of *T. bryosalmonae* by microscopic examination of cultured colonies, but subsequently detected infection by PCR and sequencing. Notably, a colony of *Plumatella fungosa* which was deployed during Trial 2 in the channel from the River Kennet became infected by worm-like stages of *Buddenbrockia plumatellae* (Canning et al. 2002). This infection was not detected until mature worm-like stages of *B. plumatellae* appeared after the *P. fungosa* colony had been in culture for 17 d. Ultrastructural examination revealed that in addition to containing mature worm-like stages, the bryozoan harboured single cell stages of *B. plumatellae* in the body wall. It thus appears that *B. plumatellae* initially infects the body wall, where it proliferates as single cells prior to developing into mature worms (Canning et al. 2002). We propose that our positive PCR results reflect the presence of similar cryptic early stages of *T. bryosalmonae* in the body wall of *Fredericella sultana*. This is further supported by the proliferation of mature sac stages of *T. bryosalmonae* after 71 d of laboratory culture in an *F. sultana* colony collected from a different river system (S. Tops & B. Okamura unpubl. data). Regular microscopic examination of this colony did not detect any sign of infection prior to the development of sacs.

Bryozoans were maintained in culture for 4 wk and could have become infected at any time during the previous 2 wk period in the field. This suggests that, under the conditions experienced, maturation of sac stages from the date of initial infection takes longer than 4 to 6 wk. Whether there is a specific cue which is required for further development of *Tetracapsuloides bryosalmonae* from cryptic stages to mature, spore-producing sacs, or whether simply a certain period is required for stages to develop in the body wall of bryozoan hosts is an important topic for future studies.

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