Molecular studies on the seasonal occurrence and development of five myxozoans in farmed *Salmo trutta* L.

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(*Received 12 May 2005; revised 20 July 2005; accepted 1 August 2005; first published online 11 October 2005*)

SUMMARY

Five myxozoan species, *Tetracapsuloides bryosalmonae*, *Sphaerospora truttae*, *Chloromyxum schurovi*, *Chloromyxum truttae* and a *Myxobolus* species were detected in farmed brown trout, *Salmo trutta* L. from Central Scotland. Using PCR and *in situ* hybridization, this study investigated the seasonal occurrence and tissue location of these species in young of the year brown trout. *C. schurovi*, *C. truttae* and *Myxobolus* sp. were first detected in brown trout in April, 2 months before *T. bryosalmonae* and *S. truttae*. *T. bryosalmonae* and *S. truttae* showed proliferation in the blood with intravascular stages of *T. bryosalmonae* accumulating in the heart. In contrast, only small amounts of PCR products of *C. schurovi* and *C. truttae* were obtained from the blood, suggesting that these species use the vascular system for transport but proliferate only in their target tissues from which large amounts of PCR product were obtained and where parasites were visible in histological sections. Large amounts of PCR product were obtained for *T. bryosalmonae, S. truttae* and both *Chloromyxum* species from the gills of brown trout, suggesting the gills as entry locus for these species. The neurotropic *Myxobolus* species formed plasmodia predominantly in the peripheral nerves, possibly indicating an entry route through the skin. Presporogonic stages of all other species had disappeared by September and mature spores were present from August onwards.

Key words: Myxozoa, *Salmo trutta*, seasonal occurrence, molecular, tissue localization.

INTRODUCTION

In temperate climates, myxozoan infections often follow seasonal patterns of abundance with high parasite burdens in the fish hosts in the summer months when the water temperatures are elevated. In salmonids, the myxozoan *Ceratomyxa shasta* Noble, 1950 was found to infect fish between April and December (Hendrickson, Carleton and Manzer, 1989) and the related intestinal disease, ceratomyxosis, generally occurs at water temperatures above 10 °C (Palenzuela and Bartholomew, 2002). Similarly, the massive proliferation of extrasporogonic stages of *Tetracapsuloides bryosalmonae* Canning *et al*. 2002, the cause of proliferative kidney disease (PKD) in salmonids, occurs only above 9 °C (Ferguson, 1981; Clifton-Hadley, Bucke and Richards, 1984). It has been suggested that the impact of temperature on the development of PKD primarily influences the kinetics of *Tetracapsuloides* multiplication in the host (Gay, Okamura and De Kinkelin, 2001) and similar conditions might apply for other myxozoan species in temperate climates. With the exeption of *T. bryosalmonae*, which produces extrasporogonic stages in large numbers, little is known about the location in the host and the seasonal occurrence of presporogonic stages of other myxozoans. The complete developmental cycle in fish has been described in detail for *Myxobolus cerebralis* Hofer, 1903 based on ultrastructural observations (El-Matbouli, Hoffmann and Mandok, 1995). The seasonal occurrence of *Sphaerospora truttae* Fischer-Scherl, El-Matbouli and Hoffmann, 1986 in fish was studied by McGeorge, Sommerville and Wootten (1994, 1996) and recently, the complete spatio-temporal development of *S. truttae* in the salmonid host was elucidated using *in situ* hybridization (ISH, Holzer, Sommerville and Wootten, 2003). This methodology, based on the detection of 18S rDNA in tissue sections, not only visualizes all developmental stages of the parasite in its biological context but also specifically identifies the myxozoan. This technique is especially useful in mixed infections. PCR amplification of 18S rDNA has been the most common molecular approach for the detection and identification of myxozoans, and protocols have been developed for various species, e.g. *C. shasta* (Bartholomew, Rodriguez and Arakawa, 1995; Palenzuela, Trobridge and Bartholomew, 1999), *M. cerebralis* (Andree, MacConnell and Hedrick, 1998; Baldwin and Myklebust, 2002) or *T. bryosalmonae* (Saulnier and De Kinkelin, 1997; Kent *et al*. 1998; Morris, Morris and Adams, 2002). Riboprinting has also been used with some success (e.g. Xiao and Desser, 2000; Eszterbauer *et al*. 2001; Hallett *et al*. 2004).

Due to the success of molecular methods for the specific detection of myxozoan species, this study used PCR and ISH for the identification, localization

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Parasitology (2006), **132**, 193–205.
doi:10.1017/S0031182005008917 Printed in the United Kingdom

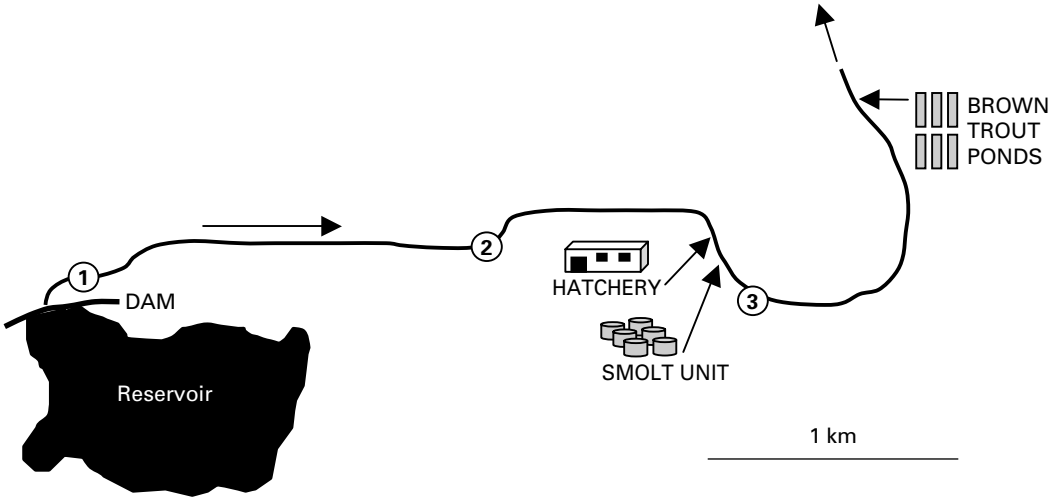

Fig. 1. Schematical illustration of the study site showing the location of the salmonid hatchery, smolt producing unit and the brown trout ponds. ① to ③ are sampling spots for wild brown trout from the stream.

and visualization of myxozoans in brown trout, *Salmo trutta* L. The aim of this study was to detect the period of infection and the presporogonic development of all the myxozoans in the fish host by monitoring farmed populations of brown trout over a year.

MATERIALS AND METHODS

Study site and sampling regime

The study site is located in Central Scotland, UK, and produces Atlantic salmon, *Salmo salar* L. smolts and brown trout. The fish farm is fed by a small stream which exits from a large reservoir of approximately 0·5 km² surface area, and it consists of 3 units, a salmon and trout hatchery, a tank system for rearing salmon smolts and 42 earthen ponds for the production of brown trout (Fig. 1). In contrast to the hatchery and the smolt unit, which receive natural stream water, water entering the pond system is a mixture of fresh stream water with water used in the rearing facilities upstream.

Eggs are derived from a broodstock population of brown trout which is maintained in the earthen ponds. In 2004, a population of brown trout was studied from the egg stage to alevin (hatchling) and fry up to a total body length of 15 cm in their first year of life (0+). These fish hatched on 10 February and were kept in stream water at the hatchery and subsequently in a larger tank at the smolt unit before they were moved to the ponds (11 000 fry per 100 m³ pond) as fry at 4 g body weight, on 21 June. Apart from the monthly samples of 0+ trout, trout in the second (1+) and in the third year of life (2+) from the pond system were examined from time to time (sampling dates are given in Table 1).

In order to compare parasite data from wild and cultured brown trout, electrofishing was conducted in the stream feeding the farm system on 26 August 2004. Three locations on the stream were fished and 6 brown trout (between 5·5 cm and 12 cm total body length) from each location were examined: (1) 3·8 km upstream of the ponds, just below the water supply reservoir, (2) 2·8 km upstream of the ponds, and (3) 1·2 km upstream of the ponds, just below the smolt unit (Fig. 1). These were considered to be wild but those caught upstream from the hatchery could have been escapees from the farm. However, if this was the case, they could not have been more than 10 weeks old when they escaped and, therefore had lived as wild for most of their life.

Samples were taken from 2-phenoxyethanol-euthanized fish. Smear preparations of all organs were observed under the microscope and whole fish (until April) or organ pieces (from May onwards) were used for DNA extraction by phenol-chloroform (Holzer, Sommerville and Wootten, 2004). A new blade was used for each organ. Adjacent organ parts or whole fish were fixed in 4% neutral buffered formalin and processed for general histology (Haematoxylin & Eosin; Pearse, 1968) and *in situ* hybridization. 6 μm paraffin sections were adhered to 3-aminopropyl-triethoxy-silane (APES) coated slides.

All myxospore types detected were measured in fresh smear preparations according to the recommendations of Lom and Arthur (1989).

Molecular techniques

For the non-specific PCR detection of any myxozoan in the different organs, a nested PCR was used which had been previously used to amplify 18S rDNA of various myxozoans (Holzer, Sommerville and Wootten, 2004). General 18S primers 18e and 18g (Hillis and Dixon, 1991) were applied in the first round and myxozoan-specific but species-unspecific primers Myxgp2F (Kent *et al.* 1998) and Act1R (Hallett, Atkinson and El-Matbouli, 2002) in the

Table 1. Sampling regime for a 0+ brown trout population and older 1+ and 2+ brown trout throughout the year 2004: numbers of fish screened for myxozoans by different examination methods

Month	Age	Location	Squash	PCR	Histo/ISH
0+ fish					
February	Eggs	Hatchery	—	20	—
February	1 wk (alevins)	Hatchery	—	22	—
March	5 wks (alevins)	Hatchery	10	18	10
April	9 wks	Hatchery	10	20	10
May	12–14 wks	Smolt unit	15	29	16
June	17–19 weeks	Smolt unit	20	20	10
July	26 weeks	Ponds	12	12	8
August	30 weeks	Ponds	14	13	10
September	34–36 weeks	Ponds	25	25	21
October	41 weeks	Ponds	12	12	8
November	45 weeks	Ponds	12	12	8
1+ fish					
March	13 months	Ponds	10	10	10
July	17 months	Ponds	4	4	2
August	18 months	Ponds	4	4	4
2+ fish					
March	25 months	Ponds	9	9	9
August	?	Ponds	1	1	1

second round using the 18e/18g PCR product at a dilution of 1:6. Specific PCR primers were used for the detection of *T. bryosalmonae*, *S. truttae*, *Chloromyxum truttae* Léger, 1906 and *Chloromyxum schurovi* Shul'man & Ieshko, 2003 based on their 18S rDNA sequences. All primers are summarized in Table 2.

PCR was performed in 10 μl volumes with 0·3 units of TITANIUM Taq DNA polymerase and 10 × buffer containing 1·5 mM $MgCl_2$ (BD Biosciences Clonetech), 0·2 mM of each dNTP, 0·5 mM of each primer, and 1 μl (10–150 ng) of template. Denaturation of DNA (95 °C for 3 min) was followed by 30 cycles of amplification (95 °C for 50 sec, annealing temperature (Table 2) for 50 sec, and 70 °C for 1 min 20 sec) and terminated by a 4 min extension (70 °C). PCR products were separated in an 1% agarose gel containing ethidium bromide in sodium boric acid buffer (Brody and Kern, 2004) at 300 V for 15 min and thereafter visualized under UV light.

In order to clarify the identity of some developmental myxosporean stages which were detected by the myxozoan specific PCR reaction (nested Myxgp2F/Act1R, see above), but which did not amplify with any of the species-specific primer pairs used, DNA sequencing was conducted. Myxgp2F/Act1R PCR products were sequenced from 3 whole fish (aged 9 weeks), 5 muscle and 5 brain samples (from fish aged 14, 26 and 36 weeks). The *Myxobolus* sp. detected in this study was sequenced from the Myxgp2F/Act1R PCR products of 2 DNA extractions of mature spores which had been separated from host tissue using polyethylene glycol and dextran (Holzer *et al.* 2003). For sequencing, the PCR products were excised from a 1% agarose gel in sodium boric acid buffer and purified using GFX PCR DNA and gel band purification spin columns (Amersham Pharmacia Biotech Inc.). Myxgp2F and Act1R were used for cycle sequencing of the 18S fragments in an ABI PRISM 377 DNA sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

For the detection of developmental myxozoan stages in tissue sections *in situ* hybridization was conducted employing one primer for each myxozoan species (see Table 2) and following a biotin-streptavidin based protocol developed for *S. truttae* (Holzer *et al.* 2003).

RESULTS

Identification of myxozoan species

During the course of the year, 5 myxozoan species were detected in brown trout. All but *T. bryosalmonae* were identified by measurements of mature spores (Table 3) and by specific PCR and/or DNA sequencing. *T. bryosalmonae* was identified by PCR and morphology of pre-sporogonic stages only. In the kidney, 3 myxozoan species, *T. bryosalmonae*, *S. truttae* and *C. schurovi* were detected which sporulated in the renal tubules and they were commonly found in mixed infections.

The myxozoan present in the gall bladders was identified as *C. truttae* and spore-forming plasmodia of a *Myxobolus* species were found in the nervous tissue, predominantly in the peripheral parts of the spinal and cranial nerves, embedded in the musculature. Spores of *Myxobolus* sp. were oval and lacked

Table 2. Primers used for PCR amplification and *in situ* hybridization (ISH) of myzozoans found in brown trout

Myxozoan species detected	Name	Sequence	PCR annealing	ISH	Reference
Any myxozoan	18e	5′-TGG TTG ATC CTG CCA GT-3′	63 °C	—	Hillis and Dixon (1991)
	18g	5′-GGT AGT AGC GAC GGG CGG TGT G-3′		—	Hillis and Dixon (1991)
	Myxgp2F	5′-TGG ATA ACC GTG GGA AA-3′	58 °C (nested)	—	Kent *et al.* (1998)
	Act1R	5′-AAT TTC ACC TCT CGC TGC CA-3′		—	Hallett *et al.* (2002)
Tetracapsuloides bryosalmonae	5F	5′-CCTATCAATGAGTAGGAGA-3′	55 °C	—	Kent *et al.* (1998)
	6R	5′-GGACCTTACTCGTTTCCGACC-3′		ISH	Kent *et al.* (1998)
Sphaerospora truttae	STRIF	5′-GATCGGTCTCAGCCCTTCT-3′	63 °C	—	This study
(*GenBank Acc. no. AJ581915*)	*STRIR*	5′-GGACACCCACTACACCCATCT-3′		ISH	Holzer *et al.* (2003)
Chloromyxum truttae	CtrutF	5′-ACGGGGTAGTTTTCTTCGGTCTGC-3′	60 ° C	—	This study
(GenBank Acc. no. AJ581916)	CtrutR	5′-TCGTGGTTGGTGTATTGTTGA-3′		ISH	This study
Chloromyxum schurovi	Neo1F	5′-TCTGGTGGGCCGTGTATGAT-3′	58 °C	—	This study
(GenBank Acc. no. AJ581917)	Neo1R	5′-TCTGTTTTTATGCTTGGTTGTCTT-3′		ISH	This study
Myxobolus sp.	Mnerv2R	5′-CGAACCTCGGACTGTCGTTC-3′	—	ISH	This study
(GenBank Acc. no. AM042702)					

sutural markings. The polar capsules were pyriform and the polar filament had 6–7 turns. The partial 18S sequence obtained from mature *Myxobolus* spores (854 bp, GenBank Accession number AM042702) did not match that of any myxozoan sequence on GenBank. The DNA sequences obtained from 5 individual brains, 5 individual muscle samples and 3 whole fry of brown trout were identical with the *Myxobolus* sp. sequence obtained from separated spores. In 4 muscle samples and in those from whole fry other myxozoan species were also amplified, but in the brains only *Myxobolus* sp. was detected. Thus the presence of the non-specific Myxgp2F/Act1R band in extractions from brains was interpreted as *Myxobolus* sp. infection.

Seasonal prevalence determined by PCR

PCR was used in order to investigate the seasonal occurrence of the different myxozoan species in 0+ brown trout and to ascertain whether 1+ and 2+brown trout maintain the infections or become re-infected.

Figure 2 summarizes the seasonal prevalences of all myxozoan species in 0+ brown trout, detected by PCR. Brown trout eggs and alevins (post-hatchlings) in the hatchery tested negative for myxozoans. Infections were first detected in brown trout fry in April, at age 9 weeks when the average water temperature was 9 °C. *Myxobolus* sp., *C. schurovi* and *C. truttae* were detected in DNA extractions from April onwards, whereas *S. truttae* and *T. bryosalmonae* were only detected 2 months later, i.e. from June onwards.

High prevalences of infection were observed soon after the first occurrence of each species. One hundred percent infection prevalence was obtained by *C. truttae*, by *Myxobolus* sp. and by *T. bryosalmonae* in July. The prevalence of *S. truttae* reached 50% in July and remained at an average of 60% thereafter. *C. schurovi* showed a similar prevalence (63%) but reached this level at the beginning of May.

In 1+ brown trout, small amounts of PCR products were obtained for *T. bryosalmonae*, indicating that the infection was still present throughout the second summer. 1+ brown trout also tested PCR positive for *S. truttae* in March but, in contrast to *T. bryosalmonae*, all kidneys tested negative for infection in July and August. *T. bryosalmonae* and *S. truttae* were never detected in 2+ brown trout. *C. truttae, C. schurovi.* and *Myxobolus* sp. were present in 1+ and 2+ trout and large amounts of PCR products were consistently obtained in these fish.

All myxozoan species detected in the farmed brown trout were also found in wild brown trout obtained from 3 sites upstream of the trout ponds (Fig. 1, Table 4). *S. truttae* and *C. schurovi.* were present in fish from all sites with overall prevalences

Table 3. Measurements of the myxosporean spores detected in 0+ *Salmo trutta* L. from central Scotland (L = larger pair of polar capsules, S = smaller pair of polar capsules.)

	Chloromyxum schurovi	*Sphaerospora truttae*	*Chloromyxum truttae*	*Myxobolus* sp.
Spore				
Length	8·4 (7·6–9·2)	7·4 (6·5–8·4)	9·7 (8·2–10·5)	8·8 (7·8–9·2)
Width	8·0 (7·7–8·5)	9·9 (8·8–11·2)	9·4 (7·9–10·5)	6·4 (5·8–7·0)
Thickness	8·1 (7·5–8·8)	8·1 (6·6–9·2)	8·9 (8·5–9·8)	5·1 (4·7–5·5)
Polar capsules				
Length	L 3·6 (3·5–3·7) S 3 (2·8–3·3)	2·4 (1·9–3·3)	L 3·9 (3·7–4·2) S 2·9 (2·4–3·8)	4·0 (3·2–4·7)
Width	L 2·9 (2·8–3·1) S 2·2 (2·1–2·2)	2·4 (1·9–3·3)	L 3·4 (3·0–3·8) S 2·6 (2·0–3·0)	2·6 (2·1–3·2)

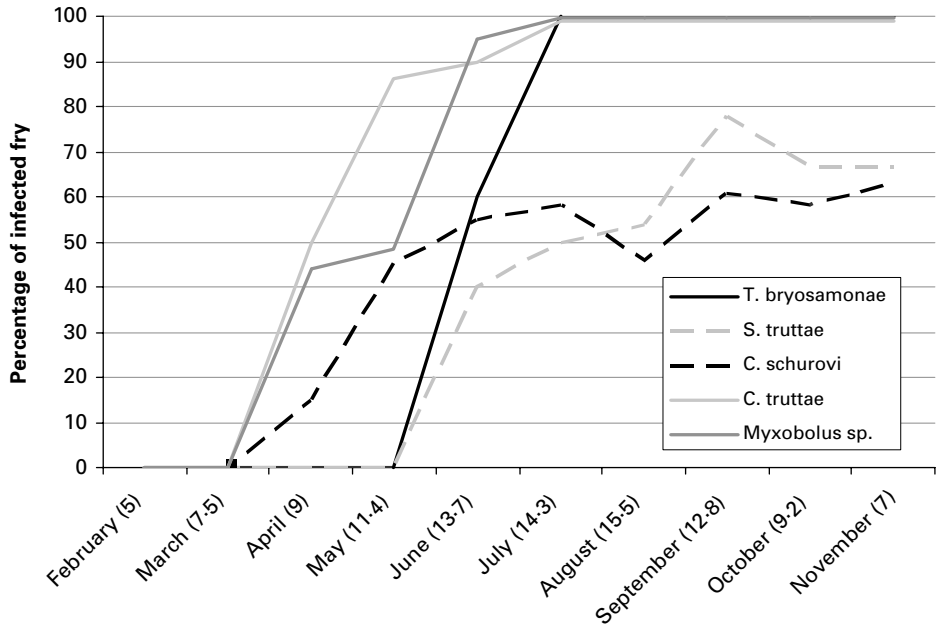

Fig. 2. Seasonal prevalence of myxozoan infections in 0+ Scottish brown trout: Values represent the percentage of brown trout that tested positive for the different myxozoan species using PCR. Numbers in parentheses behind the months provide the mean monthly water temperature in °C for each month.

of 22% and 28%, respectively. All trout from the stream were infected with *T. bryosalmonae*. In contrast to *S. truttae, C. schurovi* and *T. bryosalmonae*, *C. truttae* was not detected on the sample site below the reservoir (Fig. 1). *C. truttae* occurred in 50% of the trout from the next sampling point downstream and showed an increase in prevalence below the hatchery/smolt unit. *Myxobolus* sp. also showed an increase along the stream.

Myxozoan location in the fish using PCR, ISH and histology

Tetracapsuloides bryosalmonae. The ISH slides showed that, from June to August, small developmental stages of 1 to 8 cells with a diameter of 5 to 25 μm were present in the vascular system, predominantly in the gills and heart. In some trout, the parasites seemed to accumulate in the heart and some stages appeared to be attached to the endothelium (Fig. 3A, B). Between June and September, histozoic, pre-sporogonic stages were found in the kidney, liver and spleen, and from August until the end of the year, intratubular stages of *T. bryosalmonae* were present in 0+ trout.

Clinical proliferative kidney disease (PKD) was detected in 12% of brown trout. These fish showed minor to moderate swelling of the spleen and the kidney and histological sections showed renal interstitial hyperplasia. Numerous parasite stages were present in the spleen, the liver and kidney (Fig. 3C). Two wild brown trout (11% of all fish from the stream) also suffered from clinical PKD. In November, the brown trout in the ponds appeared to have recovered from the clinical condition and had smaller numbers of parasites but an increased

Table 4. Prevalence (percentage) of myxozoan infections in 0+ wild brown trout from the stream feeding the investigated fish farm

Location	*T. bryosalmonae*	*S. truttae*	*C. schurovi*	*C. truttae*	*Myxobolus* sp.
1 = 3·8 km upstream ponds	100	16·7	16·7	0	0
2 = 2·8 km upstream ponds	100	33·3	33·3	50	33·3
3 = 1·2 km upstream ponds/ below hatchery + smolt unit	100	16·7	16·7	66·7	100

amount of connective tissue in the kidney. Mature spores were occasionally found from September onwards.

In 1+ and 2+ brown trout only a few isolated stages of *T. bryosalmonae* were present in the epithelium and lumen of the renal tubules (Fig. 3D). Sections of tubules containing parasites and a small number of tubules with no evident parasites in the section showed a positive signal for parasite DNA/RNA in the cytoplasm of the epithelial cells, proximal to the tubular lumen (Fig. 3D). Blood and histozoic stages of *T. bryosalmonae* were never present in fish in their second summer.

Sphaerospora truttae. Gills and the blood of 0+ brown trout first tested PCR positive in June. Although positive PCR results were obtained from these organs until August, only isolated pre-sporogonic stages were present in ISH sections. These consisted of 4 up to 80 cells and occurred in the vascular system of the gills, the spleen, the liver and the interstitial tissue of the kidney. The kidneys tested PCR positive for *S. truttae* from June onwards and proliferation in the renal tubules was observed for the first time in August. Intratubular stages were present in higher numbers than pre-sporogonic ones. In 12% of the trout, the intratubular stages occurred with high intensity of infection (⩾20% of the tubules per organ section infected). These fish usually showed a mixed infection with *C. schurovi* and, although swelling of the kidney was not observed their total body length was on average 24% less than that of other trout in the ponds. Mature spores of *S. truttae* were present in samples from September onwards. The brown trout were only infected with *S. truttae* in their first year and in the following year from June onwards no infection was detected.

Chloromyxum schurovi. From April to August, PCR detected *C. schurovi* in gills, blood, kidney, spleen and liver of 0+ brown trout but from September onwards PCR amplicons were only obtained from the kidney. The amount of PCR products obtained from all organs except for some kidneys was consistently small.

The ISH procedure for *C. schurovi* could not be optimized and developmental stages of *C. schurovi* were not observed. The number of tubules infected with sporogonic stages and containing mature spores was very low in brown trout of all age classes (0–4 tubules per squash preparation). Infected tubules usually contained trophozoites in which spores were developing in disporous units. The trophozoites occurred typically together with extracellular material and pigment which appeared yellow in fresh smears (Fig. 3E) and eosinophilic when stained with H&E. Mature spores were generally present in very small numbers. In 0+ brown trout they occurred from August onwards and they were also present at all sampling dates for 1+ and 2+ trout.

In 10% of the brown trout the number of infected tubules in squashes was greater (10–15 tubules per preparation). This occurred usually in mixed infections with *S. truttae* (see above).

Chloromyxum truttae. *C. truttae* was first detected in DNA extractions from whole 0+ brown trout in April. Thereafter, large amounts of PCR products were consistently obtained from all extractions of gall bladder or bile. Frequently, livers tested PCR positive for *C. truttae*. PCR products were obtained from organs other than the gall bladder or liver in only a few fish. In 7 trout sampled between April and August, large amounts of PCR amplicons were produced in extractions from the gills and in four of these fish small amounts of PCR product was also obtained from extractions of the blood, spleens, kidneys, and on one occasion from muscle tissue. In 1+ and 2+ brown trout PCR amplicons were only obtained from gall bladders.

Early parasite stages were observed microscopically in squash preparations of the gall bladders of various 0+ brown trout. In fresh smear preparations these showed an opaque ectoplasm and a granular endoplasm (Fig. 4A). The stages were clearly motile and did not seem to be attached to the epithelium (Fig. 4B). Slides subjected to ISH showed that these early sporogonic stages are multicellular with 4–12 cells visible in histological sections (Fig. 4C). Pre-sporogonic stages of *C. truttae* were detected in very small numbers in the livers of 5 fish. In histological sections, the stages appeared isolated in the liver and consisted of 5 to 26 cells (Fig. 4D). Pre-sporogonic stages in other organs were not detected in sections.

Mature spores were first detected in 0+ brown trout in August and on all following samples they

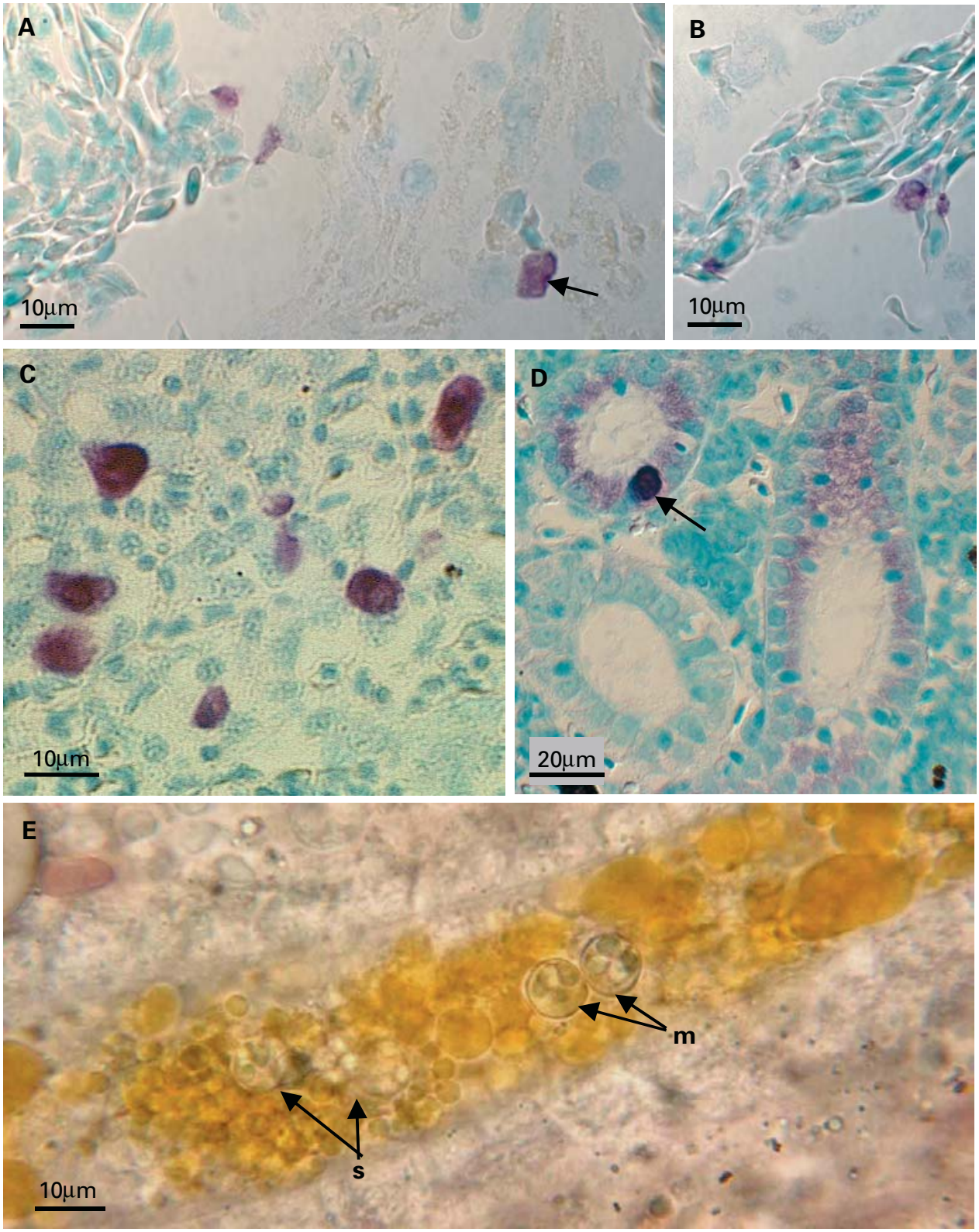

Fig. 3. (A–D) *Tetracapsuloides bryosalmonae*, ISH with parasite RNA/DNA signalling in purple. (A, B) Intravascular developmental stages in the heart; arrow indicates a stage which is attached to the endothelium. (C) Clinical PKD in the kidney of 0+ brown trout with numerous parasites in the interstitial tissue. (D) Kidney of a 1+ brown trout showing a *T. bryosalmonae* stage within the tubular epithelium (arrow) and DNA/RNA remains in the cytoplasm of previously infected epithelial cells. (E) *Chloromyxum schurovi*, fresh kidney smear showing a renal tubule filled with sporogonic stages (s), 2 mature spores (m) and extracellular material containing yellow pigment.

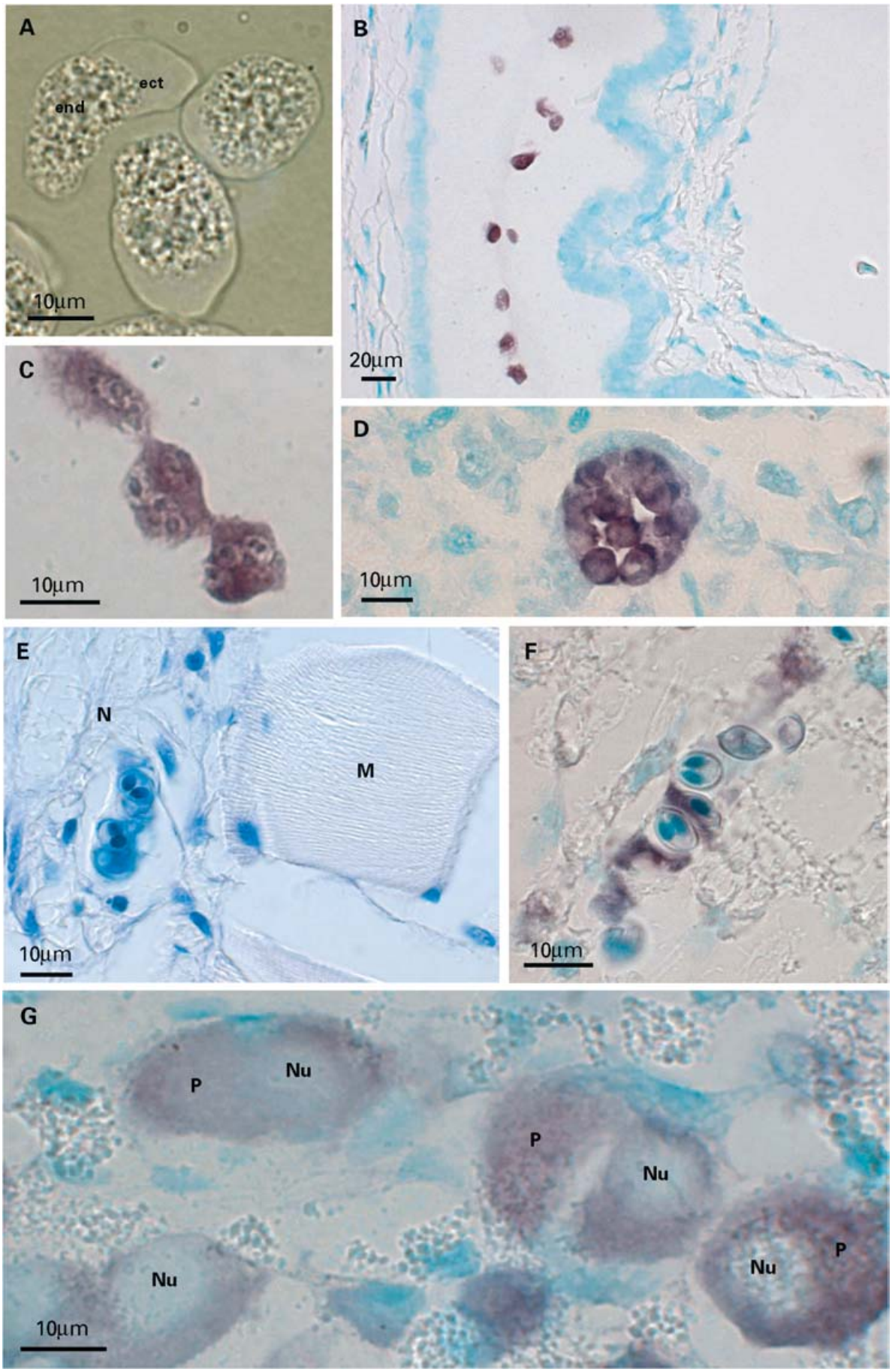

Fig. 4. For legend see opposite page.

were present in large numbers. Only a few spores were found in 1+ and 2+ trout sampled in March but their number had increased again in July and August.

Myxobolus sp. A specific PCR reaction for *Myxobolus* sp. was not developed but DNA sequencing showed that the parasite was present in the brain and the muscle. Histological sections showed that spore-forming plasmodia were located predominantly in the nervous tissue in the musculature (Fig. 4E), and were only rarely found in the spinal cord and the brain itself, although both were strongly PCR positive. In all cases, the plasmodia were elongated, up to 120 μm in length but usually only one spore to two spores wide. The shape of the plasmodia seemed to be influenced by the direction and shape of the nerve fibres and they may be located intracellularly. Individual cells constituting the plasmodium surrounding mature spores were identified using ISH (Fig. 4F). Mature spores did not stain due to insufficient digestion of their thick spore valves. Immature spores did, however, stain. Furthermore, the perikarya of many nerve cells repeatedly showed a positive signal (Fig. 4G) with the nucleus area remaining unstained. It is unclear if this is an artefact or represents parasite DNA/RNA within nerve cells which have been or are infected, however, nerve cells did not stain in control sections where no primer was used or in sections where primers for other species were applied. Plasmodia containing mature spores were first detected in August and were present until the end of the year.

Large amounts of PCR products were obtained from the brains of most 1+ and 2+ brown indicating that the infection was also present in older fish and histology revealed the presence of plasmodia containing mature spores at all sampling dates.

DISCUSSION

Myxozoan species in brown trout

Five myxozoan species were detected in cultured and wild brown trout from Central Scotland. Whereas *T. bryosalmonae, S. truttae* and *C. truttae* are well-known parasites of salmonids, *C. schurovi* has been little studied and the neurotropic *Myxobolus* species probably represents a new species.

A kidney parasite of the genus *Chloromyxum* has been mentioned in several reports from brown trout and Atlantic salmon in Europe (Sedlaczek, 1991; Holst *et al*. 1993; Feist *et al*. 2002; Shul'man and Ieshko, 2003; Holzer *et al*. 2004). The similarity of the measurements taken from mature spores suggest that all reports relate to the same species, named *Chloromyxum schurovi* Schul'man and Ieshko 2003, which has recently been re-described (Holzer *et al*. manuscript submitted) and which is the species this study refers to.

The dimensions of the spores of the neurotropic *Myxobolus* species overlapped with the measurement ranges of *Myxobolus cerebralis*, which occurs in the cartilage of various salmonids and of *Myxobolus kisutchi* Yasutake & Wood, 1957, a neurotropic parasite of *Oncorhynchus kisutch* (Walbaum, 1792). While the location of *Myxobolus* sp. differentiates the species from *M. cerebralis*, *M. kisutchi* is identical in spore size as well as location and morphology of the plasmodia (Yasutake and Wood, 1957) but has so far only been found in *O. kisutch* (Yasutake and Wood, 1957) and *Oncorhynchus tshawytscha* (Walbaum, 1792) (Wyatt, 1978) in North America. The spores of *Myxobolus* sp. are only slightly smaller than those of *Myxobolus neurobius* Schuberg and Schröder, 1905, a parasite from the nervous tissues of brown trout, but the partial 18S rDNA sequence obtained from mature spores found in this study (854 bp, GenBank Accession number AM042702) did not match that of *M. neurobius* or any other myxozoan sequence on GenBank. However, a sequence for *M. kisutchi* is not available. Based on the molecular data, *Myxobolus* sp. from this study is most closely related to *Myxobolus* sp. from the brain of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) in California (AF378342, approximately 94% sequence identity) while it is only distantly related to *M. neurobius* (AF085180, approximately 76% sequence identity) or *M. cerebralis* (U96495, approximately 77% sequence identity). Recently, a neurotropic *Myxobolus* species was detected in salmonids in Idaho (Hogge, Campbell and Johnson, 2004) and 18S rDNA sequence analysis showed that its closest relative is also the salmonid brain parasite from California. Furthermore, the location and size of plasmodia of the species from Idaho is similar to the species investigated in this study. The present *Myxobolus* sp. from brown trout might thus be identical with the species described by

Fig. 4. (A–D) *Chloromyxum truttae*. (A) Fresh smear showing motile stages in the bile with an opaque ectoplasm (ect) and a granular endoplasm (end). (B, C, D) *In situ* hybridization with parasite RNA/DNA signalling in purple. (B) Cross-section of an infected gall bladder showing motile stages without attachment to the epithelium. (C) Magnification of the motile stages showing their pluricellular structure. (D) Multicellular developmental stage of *C. truttae* in the liver. (E–G) *Myxobolus* sp. (E) Giemsa-stained section showing a typical small plasmodium (P) within a nerve (N) in the muscle (M). (F,G) *In situ* hybridization with parasite RNA/DNA signalling in purple. (F) *Plasmodium* (purple) with mature spores (do not stain due to insufficient digestion of the thick spore valves). (G) Perikarya (P) in the brain signalling the presence of parasite RNA/DNA; nuclei (Nu) are free from signal.

Hogge *et al.* (2004), however their sequence has not been published on GenBank. A new species name for *Myxobolus* sp. from brown trout cannot be given until its relation to the *Myxobolus* sp. of Hogge *et al.* (2004) or *M. kisutchi* is clarified. In order to study the present *Myxobolus* species and its relation to other members of the genus further, longer, cloned DNA fragments of *Myxobolus* sp. and all closely related species would be required. This could serve as a basis for the design of specific primers for *Myxobolus* sp., however, in using the 18S rDNA gene specific primer design could be problematic due to the high percentage of sequence similarity with some other *Myxobolus* species inhabiting the same host tissue.

Infection prevalences

In farmed and wild 0+ brown trout the prevalence of infection with *T. bryosalmonae* was 100% with clinical PKD in 12% of both farmed and wild trout. Wootten and McVicar (1982) found *T. bryosalmonae* present in 100% of brown trout in another Scottish stream, whereas Feist *et al.*(2002) found only 6–43% infection prevalence in brown trout from various rivers in England. The high prevalence of *T. bryosalmonae* in this study may reflect an abundance of bryozoan alternate hosts producing high numbers of infective stages. Extensive cover of rocks and tree roots with bryozoan colonies was detected in the stream feeding the farm (personal observation). An infection prevalence of 100% was also reached by *C. truttae* and by *Myxobolus* sp. but not by *C. schurovi* and *S. truttae*. These differences could also be explained by the abundance of alternate hosts: *Eiseniella tetraedra* (Savigny, 1826), the oligochaete host of *C. schurovi* (Holzer *et al.* 2004) prefers a gravel sediment and a habitat at the interface between water and land and thus occurred only in the stream, but not in the soft sediment of pond bottoms (personal observation). In contrast, *Stylodrilus heringianus* Claperede, 1862 the host of *Chloromyxum truttae* (Holzer *et al.* 2004), was found in the sediments of both stream and ponds. In the organically enriched sediment of the ponds, the oligochaete density is generally higher than in the natural habitat. It thus suggests that an increased production of the alternate aurantiactinomyxon stages of *C. truttae* in the ponds may be the cause of the increase of the infection prevalence in brown trout once they were transferred into the ponds, whereas levels were lower in wild fish and in farmed trout held in tanks. This hypothesis might also be indicative for the habitats of the unknown alternate hosts of *S. truttae* and *Myxobolus* sp.

Seasonal occurrence of infections and parasite locations

Initial detection of the infection with the 5 different myxozoan species detected took place between April and June and was possibly temperature dependent, although other factors such as the parasite's endogenous developmental cycle might be involved. Temperature dependency has been observed in many actinosporean types from a variety of oligochaete hosts. *C. truttae*, *C. schurovi* and *Myxobolus* sp. were able to successfully invade brown trout and proliferate in the fish at temperatures around 9 °C, 2 months before *T. bryosalmonae* and *S. truttae* occurred in the fish. The latter two species might appear later than the other myxozoans either because of the seasonal abundance of their alternate hosts or because of different temperature requirements for release from their alternate hosts. In contrast to *C. truttae*, *C. schurovi* and possibly *Myxobolus* sp., *T. bryosalmonae* does not have an oligochaete but a bryozoan alternate host and the seasonal abundance of bryozoans (Okamura *et al.* 2001) might differ from that of oligochaetes. However, all myxozoan species infected brown trout in the hatchery before they were moved to the ponds, indicating that the fish were invaded by infective spore stages from the incoming water supply.

PCR and ISH techniques have provided significant information about the pathways and proliferation sites of the myxozoans in the host. *T. bryosalmonae* and *S. truttae* show many similarities in their development in the fish host. Whereas the complete spatio-temporal cycle of development in the fish host has been described for *S. truttae* (Holzer *et al.* 2003), not all stages are known from *T. bryosalmonae*. Morris, Adams and Richards (2000) demonstrated by ISH, that *T. bryosalmonae* was present in the gills of rainbow trout 3 days after exposure of the fish to infective stages. In this study, the presence of *T. bryosalmonae* in the gills was confirmed which perhaps suggests that the gills represent the entry locus of *T. bryosalmonae* into fish, as in *S. truttae* (Holzer *et al.* 2003). While blood stages of *T. bryosalmonae* have previously been observed in blood smears, this is the first time they have been visualized using ISH: In contrast to *S. truttae*, the blood stages of *T. bryosalmonae* were small, sometimes consisting of only a single cell but never exceeding 25 μm in diameter. Accumulation of *T. bryosalmonae* blood stages in the heart represents a novel finding, and it is suggested that the parasite might be neutralised in this location. Using ISH, it was not possible to resolve the interaction between the parasite and the heart epithelium in detail, but a study using TEM could shine more light on this hypothesis. The endothelium of the fish heart has a prominent role in the endocytosis of substances circulating in the blood (Ferguson, 1975; Press and Evensen, 1999) which might explain the concentration of *T. bryosalmonae* in the heart and the attachment to the endothelium. This kind of non-specific immune reseponse against blood stages has not been observed for other

myxozoans, in the case of *S. truttae* only histozoic stages were ingested and destroyed (Holzer *et al.* 2003).

In contrast to *S. truttae* and *T. bryosalmonae*, the amount of PCR products obtained from *C. schurovi* and *C. truttae* in the blood was small. The outcome of a PCR is influenced by various factors e.g. the amount of DNA present in the sample or the presence of PCR inhibitors, however, the amount of product might be indicative of the quantity of parasites if the conditions in the DNA extraction protocol are constant at all times. Throughout this study, DNA was extracted from 6 μl of blood which should result in a similar amount of DNA obtained containing a comparable amount of PCR inhibitors. The results from PCR are supported by the observation that *C. schurovi* and *C. truttae* stages were not detected in blood vessels in sections used for ISH. Together, this evidence perhaps suggests that a small number of parasites are present in the blood and that these two species use the blood stream as means of transport rather than as a proliferation site, in contrast to *T. bryosalmonae* and *S. truttae*. Significant proliferation of *C. schurovi* and *C. truttae* takes place only once these parasites reach their target organs, i.e. the gall bladder and the kidney, respectively. PCR signals of *C. schurovi* and *C. truttae* were obtained from various organs but only in fish in which these parasites were present in the blood, thus indicating an intravascular but not histozoic presence in these organs. It may be suggested that both species enter their fish host predominantly through the gills as strong PCR signals were detected in the gills of individual fish, from where they may be transported to the target organs via the blood. In the case of *C. truttae*, some intracellular histozoic stages were detected in sections of the liver indicating the exit route of the parasite from the capillary network into the interstitial space of the liver. Thereafter *C. truttae* probably enters the gall bladder through the bile collecting ducts.

In contrast to the other myxozoans, little information was obtained concerning the seasonal localisation of *Myxobolus* sp. From the first finding in March the parasite was present in the nervous tissue. The presence of spore-forming plasmodia predominantly in the distal parts of the nervous system in the muscle and the cranium but not in the central nervous system suggests that the infection initiated in the periphery, i.e. the skin of brown trout. *M. cerebralis* has been shown to invade rainbow trout via the mucous cells in the skin (El-Matbouli *et al.* 1995; El-Matbouli *et al.* 1999) and then to migrate intercellularly within nerves in order to reach the target tissue, the cartilage (El-Matbouli *et al.* 1995). Although plasmodia of *Myxobolus* sp. were rare in the central nervous system, brains tested PCR positive in 100% of brown trout. This might be due to intracellularly infected nerve cells whose perikarya lie in the brain, which would also explain the ISH signal in these cells.

Remarkable differences in the patterns of spore production in the brown trout were observed between the myxozoan species. *T. bryosalmonae* and *S. truttae* began spore formation in September and all spores were apparently shed by the following spring. Brown trout acquires immunity towards these species (Foott and Hedrick, 1987; McGeorge, 1994). *C. schurovi* and *C. truttae* mature spores were first detected in August and over the following winter in 0+ brown trout. In the following year spore production continued, presumably by proliferation of overwintered developmental stages in the kidney tubules and the gall bladders, respectively, as well as re-infection. Plasmodia of *Hoferellus gilsoni* (Debaisieux, 1925) have been shown to overwinter in the urinary bladder of American eels, *Anguilla rostrata* Lesueur, 1817 (Melendy and Cone, 2001). However, in species against which immunity is not acquired, re-infection cannot be excluded. Lom, Molnar and Dyková (1986) found *H. gilsoni* in *Anguilla anguilla* (L.) to be produced throughout the year and year-round production of spores was also observed in *C. schurovi* in this study. The *Chloromyxum* species thus show a less pronounced seasonal profile than *T. bryosalmonae* and *S. truttae*. The nerve-dwelling *Myxobolus* species produced spores from August onwards but due to the lack of an *in vivo* exit route, spores are presumably not dispersed until the host fish dies.

This study has provided novel information about the prevalences and locations of 5 different myxozoan species in Scottish brown trout. This information adds to the understanding of different myxozoan infection strategies and their seasonal proliferation and pathways in fish, thus allowing implications for aquaculture strategies which target specific stages during defined periods of the year.

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