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Proliferative kidney disease in rainbow trout: time- and temperature-related renal pathology and parasite distribution

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ABSTRACT: Proliferative kidney disease is a parasitic infection of salmonid fishes caused by *Tetracapsuloides bryosalmonae.* The main target organ of the parasite in the fish is the kidney. To investigate the influence of water temperature on the disease in fish, rainbow trout *Oncorhynchus mykiss* infected with *T. bryosalmonae* were kept at 12°C and 18°C. The number of parasites, the type and degree of lesions in the kidney and the mortality rate was evaluated from infection until full development of disease. While mortality stayed low at 12°C, it reached 77% at 18°C. At 12°C, pathological lesions were dominated by a multifocal proliferative and granulomatous interstitial nephritis. This was accompanied by low numbers of *T. bryosalmonae*, mainly located in the interstitial lesions. With progression of the disease, small numbers of parasites appeared in the excretory tubuli, and parasite DNA was detected in the urine. Parasite degeneration in the interstitium was observed at late stages of the disease. At 18°C, pathological lesions in kidneys were more severe and more widely distributed, and accompanied by significantly higher parasite numbers. Distribution of parasites in the renal compartments, onset of parasite degeneration and time course of appearance of parasite DNA in urine were not clearly different from the 12°C group. These findings indicate that higher mortality at 18°C compared to 12°C is associated with an enhanced severity of renal pathology and increased parasite numbers.

KEY WORDS: *Tetracapsuloides bryosalmonae* · Kidney histopathology · Temperature · Proliferative kidney disease · Rainbow trout · Parasite localization

INTRODUCTION

Proliferative kidney disease (PKD) is a parasitic infection of salmonid fishes caused by *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) (Feist & Bucke 1993, Hedrick et al. 1993, Canning et al. 2000, Okamura et al. 2001). Clinical disease leads to high mortalities in affected fish (Ferguson & Ball 1979, Clifton-Hadley et al. 1986). PKD is discussed as one factor contributing to the decrease of wild salmonid populations in Switzerland (Wahli et al. 2002, 2007, Burkhardt-Holm et al. 2005) and Norway (Sterud et al. 2007).

The parasite life-cycle, as far as known to date, includes different species of bryozoans as invertebrate hosts (Anderson et al. 1999, Longshaw et al. 1999, Okamura et al. 2001), and salmonids as vertebrate hosts (Feist & Bucke 1993, Hedrick et al. 1993). *Tetracapsuloides bryosalmonae* infects the fish through skin and gills (Feist et al. 2001, Longshaw et al. 2002) and after systemic circulation enters the main target organ, the kidney (Kent & Hedrick 1985). In the kidney *T. bryosalmonae* undergoes multiplication and differentiation from extrasporogonic to sporogonic stages (Kent & Hedrick 1985). Infected salmonids can probably excrete spores via the urine (Kent & Hedrick 1985, Hedrick et al. 2004) and spores excreted by brown trout *Salmo trutta* or brook trout *Salvelinus fontinalis* can reinfect bryozoans (Morris & Adams 2006, Grabner & El-Matbouli 2008).

The disease caused by *Tetracapsuloides bryosalmonae* is temperature dependent, as clinical signs

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and PKD related mortality increase at water temperatures above 15°C, whereas below 15°C low mortality rates are reported (Ferguson & Ball 1979, Ferguson 1981, Clifton-Hadley et al. 1986). In parallel with the occurrence of clinical disease signs at water temperatures above 15°C, severe pathological lesions are observed in infected fish (Kent & Hedrick 1985, Clifton-Hadley et al. 1987, Morris et al. 2005). Currently it is not known whether and how parasite development in the fish and the pathological response to the infection change with temperature. Such knowledge could be important for understanding the reasons for the increased mortality of PKD-infected fish at elevated water temperatures. As the most marked changes in *T. bryosalmonae* infected fish are found in the kidney (Kent & Hedrick 1985, Clifton-Hadley et al. 1987, Morris et al. 2005), this study concentrated on renal disease.

The aim of the present study was to investigate and compare renal pathology as well as parasite localization in different renal compartments including possible transfer into the urine at 2 temperatures, 12°C and 18°C, which are characterized by different levels of clinical disease manifestation and mortality. The sequence of pathological changes in kidneys of infected fish was studied by histopathology. The localization of parasites in the kidney was determined by histology, immunohistochemistry and *in situ* hybridization. Urinary excretion of parasite DNA was assessed by real-time PCR.

MATERIALS AND METHODS

Experimental exposure and sampling. This study used 425 0+ rainbow trout *Oncorhynchus mykiss* (mean size of 8.3 ± 0.52 cm) originating from a commercial trout farm with water temperature below 12°C and no history of PKD. Before exposure, 5 randomly sampled fish were subjected to macroscopic and histological examinations for signs of PKD and tested for the presence of *Tetracapsuloides bryosalmonae* by real-time PCR. The remaining 420 trout were exposed in September to water from a river with water temperature ranging from 12 to 16°C, regularly shown to harbor *T. bryosalmonae*-infected fish in previous years. Five days after the start of exposure 20 randomly sampled fish were investigated for the presence of *T. bryosalmonae* using real-time PCR. The remaining trout were transferred to the laboratory at the Centre for Fish and Wildlife Health (FIWI) and split into 2 groups consisting of 200 fish. Fish were kept in 100 l tanks with a flow-through system and constant aeration. The transfer to the experimental temperatures of 12°C and 18°C was carried out over 1 d. Oxygen concentration in all tanks was ≥8 mg l^{-1} during the experimental period. Fish were fed commercial trout pellets (HOKOVIT, Bützberg) with a daily food ratio of 1 to 2% of body weight. Mortalities were recorded daily and dead fish were subjected to parasitological and bacteriological examination and a complete necropsy as described below. Fish from the same trout farm without exposure to the river were held as negative controls at 12°C and 18°C in the laboratory at the FIWI. These fish were not infected with *T. bryosalmonae*, as measured by repeated macroscopic examinations and by real-time PCR.

In each experimental group, 5 samples with 10 fish were taken at weekly or 14 d intervals for 6 wk, resulting in 7 samples for each group (Table 1). Fish were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories). A standard necropsy was performed on all individuals. Fresh mounts of skin and gill samples and the intestinal content were examined microscopically for the presence of parasites. For bacteriological examination, samples from liver, spleen and kidney were cultured on blood agar plates (Bio Merieux) and bromothymol-blue-lactose-agar plates. Fish were examined for external darkening, exophthalmia, anemic gills, kidney swelling, or ascites. Kidneys were removed and dissected vertically. One half was fixed in 10% buffered formalin for histopathological and immunohistochemical examination (IHC) and *in situ* hybridization (ISH). The other half of the kidney was frozen in liquid nitrogen for real-time PCR analyses.

After river water exposure, fish showed an infection with *Ichthyophthirius multifilis* until Day 19. These fish

Table 1. *Tetracapsuloides bryosalmonae*-infection experiment. Sampling schedule. From Day 0 to Day 5 post-exposure (pe) rainbow trout *Oncorhynchus mykiss* were exposed to river water known to harbour *T. bryosalmonae*. After transfer into the laboratory, trout were split into 2 groups and held in tap water at 12°C or 18°C. IHC: immunohistochemistry; ISH: *in situ* hybridization. Values indicate number of fish investigated using each analytical technique

Day pe	Temperature (°C)	Total fish sampled	Real-time PCR	IHC	ISH
0	<12	5	5	0	0
5	12–16	20	20	10	8
12	12	10	10	5	5
	18	10	10	5	4
19	12	10	10	5	4
	18	10	10	5	5
26	12	10	10	5	5
	18	10	10	6	6
33	12	10	10	5	5
	18	10	10	4	5
47	12	10	10	5	5
	18	10	10	5	5

were treated with 3% NaCl in a bath-treatment and no mortalities related to *I. multifiliis* were recorded.

To analyse fish urine for the presence of parasite DNA, at each sampling, 2 batches of 5 fish of each group were held for 30 min in 2 l tanks containing tap water with no flow through. The 30 min incubation period was selected on the basis of physiological data indicating that salmonid fish release urine approximately each 30 min (Curtis & Wood 1991). After removing fish, water was filtered through a 4 to 12 µm filter paper mesh (Schleicher & Schuell MicroScience). The filter was stored at –20°C until further analysis.

Histopathology, immunohistochemistry and *in situ* hybridization. Fixed samples were paraffin-embedded and 3 consecutive sections of 3 µm thickness were prepared. The first slide was stained with haematoxylin-eosin (H&E) for histopathological examination. The second slide was used for IHC staining and the third slide for ISH.

H&E stained slides were examined by a Zeiss-KF2 light microscope (Zeiss). Histopathological changes of the whole kidney section were classified from 0 (no alterations) to +++ (severe alterations as infiltration with high numbers of macrophages, multiple areas of haemorrhage, severe proliferation of the haematopoietic tissue, widespread necrosis or vasculitis with some thrombi formations).

For IHC, a monoclonal anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics) was used. Staining was done according to the protocol of Adams et al. (1992) with minor modifications. Unstained sections were incubated overnight using an antibody dilution of 1/100. Non-specific background staining was blocked with goat serum. A biotin-streptavidin-horseradish peroxidase staining kit (Kit Dako LSAB 2 System HRP Code Nr. K0675; DakoCytomation) followed by AEC (amino-ethyl-carbazole) staining (Dako AEC K3464) was used to visualize antibody–antigen complexes. Counterstaining of these sections was not performed. Kidney tissue of a fish known to be PKD-positive was used as a positive control. Slides incubated without the first antibody were used as negative controls.

For ISH, digoxigenin-labelled probes were produced according to Longshaw et al. (2002). ISH of DNA probes to *Tetracapsuloides bryosalmonae* was performed according to the protocol published by Longshaw et al. (2002) with minor modifications. After overnight incubation at 42°C, the hybridization chambers were removed and slides were washed in 2× SSC for 20 min, followed by exposure to 0.1× SSC at 42°C for 20 min and an additional washing step in TBS. Non-specific binding was blocked by incubation with 6% skimmed milk for 1 h. The next steps followed the protocol published by Longshaw et al. (2002). The slides were counterstained with Bismarck Brown Y for 1 min. Two slides with kidney of a fish known to be PKD-positive were used as controls, one slide as a positive control with the digoxigenin-labelled probe, the other slide as negative control using a non-digoxigenin-labelled probe in the hybridization buffer. For evaluation of IHC and ISH, 6 viewing fields (magnification: 160×) per slide and fish were randomly selected and the number of parasites were counted using light microscopy on a Zeiss-KF2 light microscope. Parasites were grouped according to location (renal interstitial tissue, vessels, tubules). For each time point the mean value for each location of all examined fish per temperature group was used.

Real-time PCR for detection of parasite DNA in fish urine and kidney tissue. For extracting genomic DNA from fish urine, the uppermost layer of a filter (used for 5 fish each) was removed with a scalpel blade. Genomic DNA including parasite DNA was extracted from that filter layer with DNAzol (Lucerna). For detection of parasite DNA in the kidneys, homogenized kidney tissue was used for genomic DNA extraction with DNAzol. The yield obtained from all samples was determined by spectrophotometry using a NanoDrop photometer (NanoDrop Technologies).

A 435 bp nucleotide sequence of the 18S rDNA gene of *Tetracapsuloides bryosalmonae* was chosen from GenBank (Accession No. AF190669; Canning et al. 1999) to be cloned into the pCR®-TOPO® vector using the TOPO TA Cloning® Kit (Invitrogen) following the manufacturer's protocol. The vector plasmid DNA was purified with the QIAprep Miniprep® kit (Qiagen) and resuspended in 8 mM NaOH. The yield was determined by spectrophotometry using the NanoDrop photometer. The plasmid DNA was amplified and sequenced in order to verify the sequence of the insert.

Forward and reverse primers were designed to amplify a 73 bp region of the 18S rDNA sequence of *Tetracapsuloides bryosalmonae*. Primers and probe (Table 2) were constructed by Microsynth (Balgach). The TaqMan probe was labeled at the 5′ end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). To test the specificity of the amplification of the probe and primer combination, a

Table 2. *Tetracapsuloides bryosalmonae*-primers and probe. Sequences used for real-time PCR. bp: base pairs

Primer/probe	Sequence (5′–3′)	Size (bp)
PKDtaqf1	GCGAGATTTGTTGCATTTAAAAAG	24
PKDtaqr1	GCACATGCAGTGTCCAATCG	20
ProbePKD	CAAAATTGTGGAACCGTCCGACTACGA	27

conventional PCR according to Kent et al. (1998) was performed and the products were checked on an agarose gel for amplification and molecular weight. The quantitative real-time PCR amplification was performed as follows: the reaction volume of 25 µl containing 1× TaqMan universal Master Mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, 200 nM fluorescent labeled probe, and 2 µg extracted DNA from the trout kidney as template. Amplification was done in an Mx4000® Multiplex Quantitative PCR System (Stratagene). The amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. All samples were analysed in duplicate.

The cloned region of the 18S rDNA gene of *Tetracapsuloides bryosalmonae* was used for the generation of standard curves. To calculate the copy numbers of the standard curve, nucleotide length and plasmid concentration were used (Yin et al. 2001). From a stock solution, a 10-fold serial dilution in buffer (8 mM NaOH) was prepared, subjected to real-time PCR and the obtained threshold cycle values (C_t) were plotted against the quantity of the plasmid DNA. The assay conditions to measure the samples of the standard curve were identical to those used for the fish samples. The regression equation obtained for the standard curve of the 18S rDNA of *T. bryosalmonae* was then used to convert the measured C_t value of an unknown sample into copy numbers of parasite DNA. A reference sample with known copy numbers of plasmid DNA was measured together with the samples to calibrate each run. The PCR product of the reference sample was sequenced to verify the specificity of the real-time PCR.

Statistical analyses. Using IHC or ISH, the parasite stages in the different locations of the kidney were counted at all sampling points in the 2 groups (12°C and 18°C treatment) by evaluation of 6 randomly selected fields on each slide. The results were tested for normal distribution with the Skewness, Kurtosis and Omnibus Normality tests. The 2 groups (12°C and 18°C group) were compared to each other at each sampling point and for differences between IHC and ISH counts. Both methods were also compared to each other. As the values were not normally distributed, we used the Mann-Whitney *U* or Wilcoxon rank-sum test to analyze for significant differences. Cumulative mortality was calculated using the number of fish that died during the experiment minus the ones used for sampling. The cumulative mortalities of the different groups were compared and tested for significant differences using the chi-square test. For all statistical tests, NCSS 2001 (Hintze 2006) was used.

RESULTS

Mortality

In the infected groups, all fish at all sampling points tested positive by means of real-time PCR for *Tetracapsuloides bryosalmonae* (100% prevalence). The cumulative mortality of fish was significantly different between the temperature groups ($p \leq 0.01$) (Fig. 1). In the group kept at 12°C, 5.6% died, while at 18°C cumulative mortality reached 77.1% at the end of the experiment. Parasitological examination of dead fish revealed no external or intestinal parasites. In most cases no bacteria could be isolated from samples from the kidney, spleen and liver examined by bacteriology. Sporadically, single colonies of mixed bacteria were isolated, but were not considered as a contributing factor to mortality. Histopathological examination revealed no changes other than those due to infection with *T. bryosalmonae*.

Pathological lesions and parasite distribution

At Day 0, before exposure to river water, no macroscopic or histological changes in the kidney were observed and no *Tetracapsuloides bryosalmonae* DNA was detectable with real-time PCR in the 5 fish sampled. After the 5 d exposure to river water all examined rainbow trout kidneys (n = 20) were confirmed positive for *T. bryosalmonae* by real-time PCR. At this time point, 2 out of 20 fish sampled showed macroscopically slightly enlarged kidneys. Histologically we observed renal haematopoietic hyperplasia and mild multifocal infiltration (mainly macrophages). In all fish sampled, small numbers of single-cell parasites were found by H&E in renal blood vessels and in the haematopoietic interstitial tissue of the kidney. The presence of a small number

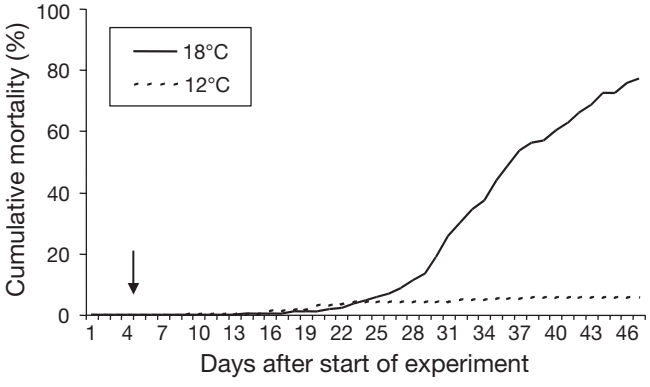

Fig. 1. *Tetracapsuloides bryosalmonae*-induced cumulative mortality of rainbow trout *Oncorhynchus mykiss* kept at 12°C and 18°C. Arrow indicates time point when trout were separated into 2 groups

Table 3. *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss* held at 12°C and 18°C. Comparison of mean parasite numbers assessed on immunohistochemistry (IHC)-stained and *in situ* hybridization (ISH)-stained slides in all kidney compartments. Numbers in brackets show lowest and highest values of parasites counted in the 6 areas per fish per sample point. *Significant difference to the temporal previous sample ($p \leq 0.05$). ^{a}Significant difference between the 2 methods ($p \leq 0.05$) used on the same samples. ^{b}Significant difference between temperatures at the same sample point and with the same method ($p \leq 0.05$). pe: post exposure

Day pe	Vessels				Interstitium				Tubules			
	IHC		ISH		IHC		ISH		IHC		ISH	
	12°C	18°C	12°C	18°C	12°C	18°C	12°C	18°C	12°C	18°C	12°C	18°C
5	0.05 (0–2)	0.05 (0–2)	0.10 (0–2)	0.10 (0–2)	0.07 (0–1)	0.07 (0–1)	1.15^{a} (0–7)	1.15^{a} (0–7)	0.00 (0–0)	0.00 (0–0)	0.00 (0–0)	0.00 (0–0)
12	0.00 (0–0)	1.33$^{\star b}$ (0–20)	0.07 (0–1)	0.08 (0–2)	0.03 (0–1)	5.30$^{\star b}$ (0–59)	0.73^{a} (0–5)	11.79$^{\star ab}$ (0–89)	0.00 (0–0)	0.03 (0–1)	0.00 (0–0)	0.25 (0–2)
19	0.23* (0–3)	1.23 (0–15)	1.38* (0–12)	2.27* (0–11)	1.00* (0–6)	6.67 (0–65)	1.75 (0–11)	18.73^{ab} (1–84)	0.00 (0–0)	0.00 (0–0)	0.25 (0–6)	0.03 (0–1)
26	0.63 (0–6)	4.08$^{\star b}$ (0–38)	0.23 (0–4)	6.50^{b} (0–29)	0.47 (0–3)	4.67^{b} (0–31)	3.07 (0–19)	84.64$^{\star ab}$ (24–171)	0.03 (0–1)	0.06 (0–1)	0.03 (0–1)	0.17 (0–2)
33	2.50* (0–15)	9.83$^{\star b}$ (0–37)	0.33 (0–6)	7.50^{b} (0–47)	7.57* (0–37)	48.25$^{\star b}$ (3–100)	12.30* (0–64)	115.54^{ab} (6–257)	0.00 (0–0)	0.04 (0–1)	0.30 (0–1)	0.79^{a} (0–4)
47	0.97 (0–6)	4.27 (0–30)	1.17 (0–9)	4.93 (0–23)	4.87 (0–14)	52.00^{b} (0–200)	7.40 (1–30)	103.73^{ab} (0–227)	0.10 (0–2)	0.10 (0–1)	0.07 (0–2)	0.60^{a} (0–3)

of parasites in renal tissue at Day 5 was confirmed by IHC and ISH (Table 3).

12°C group

A first sampling after transfer from river water to the laboratory was made at Day 12 post-exposure (pe) (i.e. 7 d after transfer from field to laboratory). At this time point, 3 out of 10 sampled fish displayed macroscopic signs of PKD, including renal hyperplasia and grayish discoloration of the kidney (Table 4). The frequency of fish displaying macroscopic signs of PKD showed a continuous increase over the study period and reached 90% at the end of the experiment. Histopathological lesions were present in all examined fish from Day 12 onwards. The most prominent histopathological lesions were seen in the interstitial haematopoietic tissue. At Day 12, few areas with mild proliferation of the haematopoietic tissue and mild infiltration mainly by macrophages were scattered throughout the interstitial tissue. Towards Day 47 these scattered, small areas progressed to a severe multifocal to coalescent proliferative and granulomatous interstitial nephritis with small, poorly circumscribed areas of necrosis (Table 4). The interstitial lesions remained in a patchy distribution until the end of the experiment. Although low numbers of parasites

Table 4. *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss* held at 12°C. Results of macroscopic and histological examination of the kidneys and presence of parasite DNA in the urine measured by real-time PCR. (+): scattered; +: mild alterations; ++: moderate alterations; +++: severe alterations; nd: not done

	Day post-exposure				
	12	19	26	33	47
Macroscopic enlargement of the kidneys (n = 10)					
Abundance	+	+	+ to ++	+ to ++	+ to ++
Prevalence (%)	30	60	70	60	90
Histological changes to the kidneys (n = 5)					
Prevalence (%)	100	100	100	100	100
Interstitium					
Proliferation of haematopoietic tissue	+	++	++	++	+
Infiltration	+	+	+	++	++
Necrosis		+	+	++	+
Haemorrhage					+
Single-cell parasites	+				
Parasites with daughter cells		+	+	++	++
Degenerating parasites					+
Vessels					
Hypertrophy of endothelial cells				+	++
Attachment of inflammatory cells			+	++	+++
Single-cell parasites in lumen	+	+			
Parasites with daughter cells in lumen			+	+	+
Degenerating parasites					+
Tubules					
Tubulonephrosis				+	+
Intraluminal stages of parasites		(+)	(+)	(+)	(+)
Real-time PCR	nd	neg	pos	pos	pos

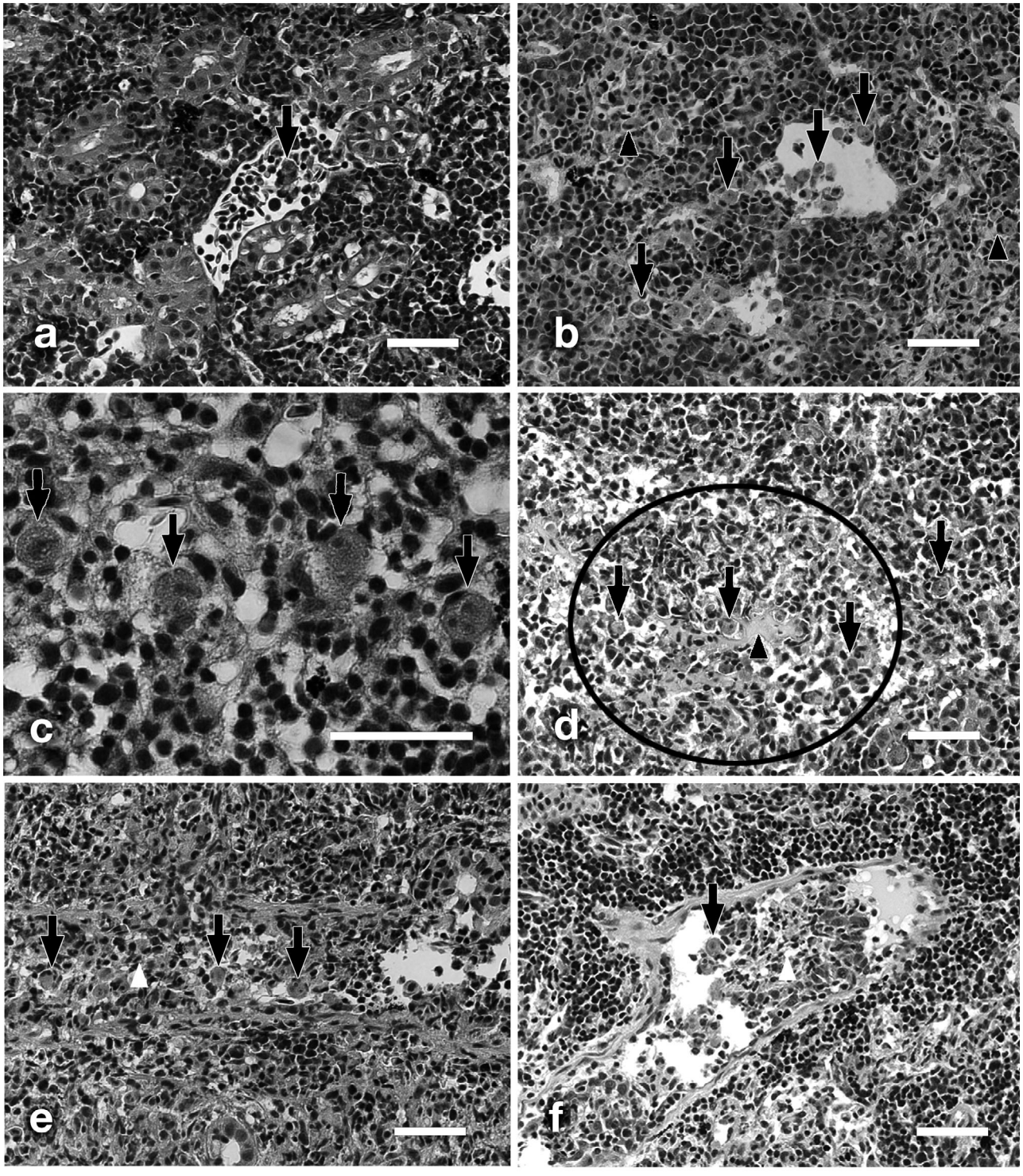

Fig. 2. *Tetracapsuloides bryosalmonae*-induced histological changes and parasite differentiation in rainbow trout *Oncorhynchus mykiss* held at (a–c) 12°C and (d–f) 18°C. (a) Parasites (arrow) in an unaltered vessel at Day 19, interstitium unaltered. (b) Parasites (arrows) penetrating vessel wall and attachment of macrophages to parasites in the vessel at Day 26. Interstitium is infiltrated with macrophages and lymphocytes (black arrowheads). (c) Degenerating parasites (arrows) in the interstitium at Day 47. (d) Severe necrosis (black circle) of the interstitium at Day 19, parasites in the interstitium (arrows), and accumulation of fibrin in the necrotic area (black arrowhead). (e) Parasites (arrows) in a severely damaged vessel; lumen filled with a thrombus (white arrowhead) at Day 26. (f) Parasites (arrow) in a vessel; lumen filled with a thrombus (white arrowhead) at Day 47. All pictures are taken from slides stained with H&E. Magnification = (a, b, d–f) 400×, (c) 1000×. All scale bars = 50 µm

were present in the vessels at all sampling points (Fig. 2a), it took until Day 26 to develop hypertrophy of endothelial cells and a mild infiltration with macrophages and lymphocytes in the vessel walls. From Day 26 until the end of the experiment macrophages and lymphocytes surrounded intravascular parasites (Fig. 2b). Tubular lesions, such as dissociation and degeneration of epithelial cells, developed after 33 d. These degenerative tubular lesions were independent of the presence of intraluminal parasites. Histologically, *Tetracapsuloides bryosalmonae* stages were visible with H&E staining at all sampling points in the renal interstitium and vessels, and after 19 d also in the tubular epithelium (Fig. 3a) and tubular lumen (Fig. 3b). While up to Day 12 only single-cell parasites were seen in infected fish, parasites with daughter cells were present at Day 19 and later. In parallel to this change in the parasite status, advanced pathological changes developed in the interstitium. At 47 dpe some parasites in the interstitium and the vessels showed signs of degeneration, such as loss of structural integrity and hypereosinophilia (Fig. 2c). IHC and ISH revealed higher parasite numbers in the interstitium compared to the other kidney compartments at all sampling points. However, the total number remained low (Table 3). At Days 5 and 12, the parasite number in the interstitium as detected by ISH was significantly higher than that assessed by IHC ($p \geq 0.05$). At all other time points no significant differences between the 2 methods were seen, neither in vessels nor interstitium, or in tubules. By means of IHC and ISH low numbers of parasites were found in the tubules beginning from Day 19 to the end of the experiment (Table 3, Fig. 3c,d). Parasite DNA in the urine was detectable from Day 26 to Day 47 pe (Table 4).

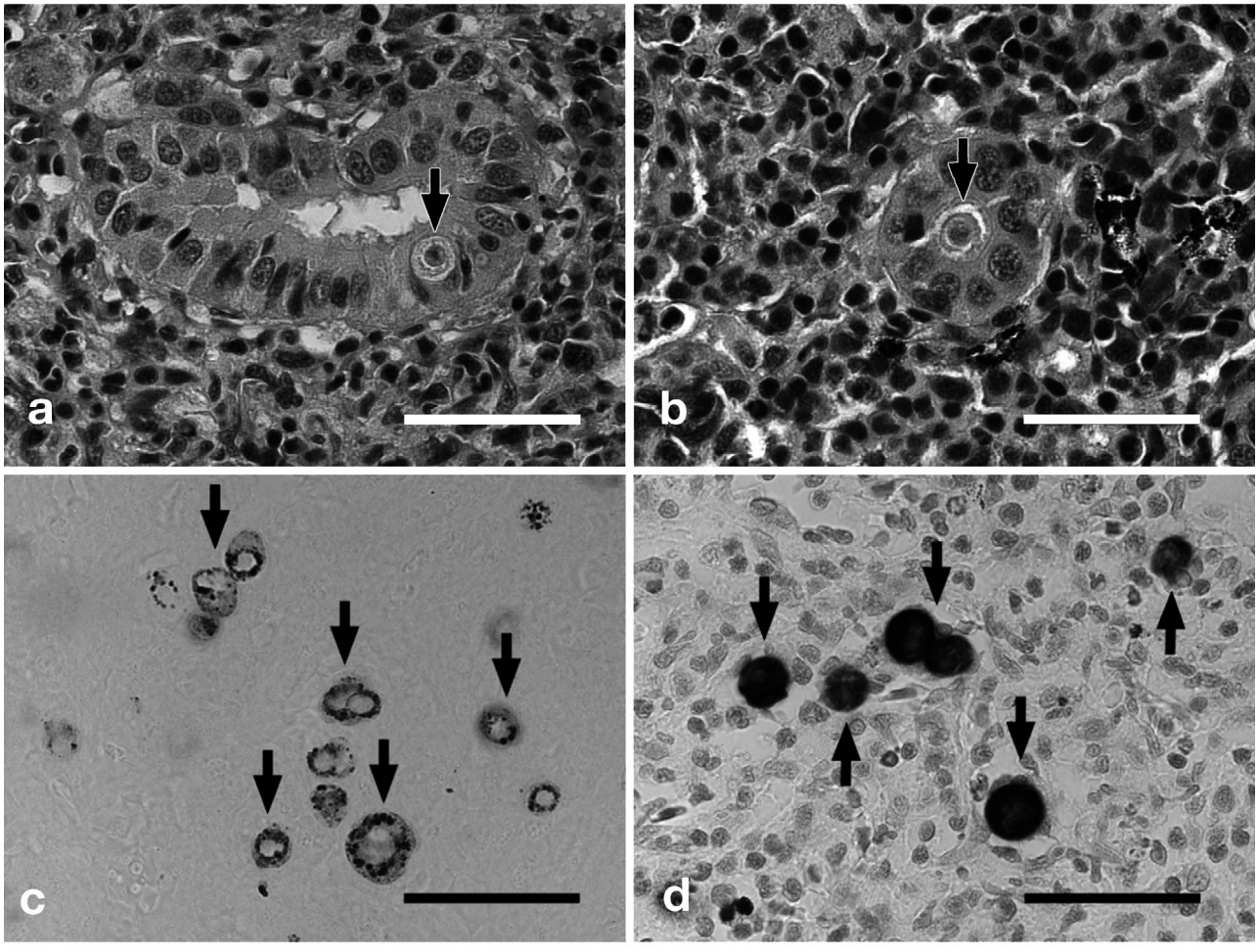

Fig. 3. *Tetracapsuloides bryosalmonae* in kidneys of infected rainbow trout *Oncorhynchus mykiss* held at 18°C. (a) Tubular wall containing a parasite at Day 47 (arrow). (b) Tubular lumen containing a parasite at Day 47 (arrow). (c) Immunohistochemical staining of parasites (arrows) in the interstitium at Day 47. (d) *In situ* hybridization of the DNA of the parasites in the interstitium (arrows) at Day 26. (a) and (b) were taken from slides stained with H&E. Magnification = 1000×. All scale bars = 50 µm

18°C group

At 18°C macroscopic lesions were generally more pronounced than in fish kept at 12°C. The prevalence of fish with macroscopic signs increased to 100% at Day 26 and remained at this level until the end of the experiment (Table 5). In the H&E stained sections, the type of renal lesions was comparable to that of the 12°C group. However, fish kept at the higher water temperature showed more severe interstitial and vascular lesions that developed more rapidly. In the haematopoietic tissue a proliferative and granulomatous interstitial nephritis with single cell necrosis was already present 12 dpe. After 19 d there were multiple poorly circumscribed areas of necrosis in the interstitial tissue (Fig. 2d). Beginning at Day 26 there were large areas of haemorrhage indicating severe vessel damage. The vessel lesions developed earlier and were more prominent compared to the 12°C group. After 19 d endothelial cells were hypertrophied and there was a mild lymphohistiocytic vasculitis. Lesions developed into a severe necrotizing vasculitis with thrombus formation from Day 26 until the end of the experiment (Table 5, Fig. 2e,f). Within the same period, parasites were penetrating through the vessel walls and macrophages and lymphocytes surrounded intravascular parasites. In contrast to the more pronounced interstitial and vascular lesions, tubular lesions were similar to those in the 12°C group with only mild tubulonephrosis.

Corresponding to the more advanced histopathological lesions in interstitium and vessels, parasites with daughter cells were already visible at Day 12. By means of IHC and ISH, the localization of parasites was comparable to the 12°C group with highest parasite numbers in the interstitium (Table 2, Fig. 3c,d). The number of parasites in the vessels as detectable by both IHC and ISH was significantly higher in the 18°C group compared with the 12°C group at Days 26 and 33 ($p \leq 0.05$). The number of parasites in the interstitium was about 14 to 15 times higher compared to the 12°C group with a significant difference to the 12°C group at Days 12, 26, 33 and 47 ($p \leq 0.05$; Table 2). Numbers of parasites in tubules remained low over the whole experimental period, both in the 12°C group and in the 18°C group. Small numbers of parasites were detected by ISH in the tubules by Day 12. The number of parasites found by IHC and ISH revealed significant differences between the 2 methods at all sampling points in the interstitium ($p \leq 0.05$) and at Days 33 and 47 in the lumen of the tubules ($p \leq 0.05$) with more parasites detected by ISH. In the urine, parasite DNA was detectable at Days 26 and 47 (Table 4), similar to the 12°C group.

Table 5. *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss* held at 18°C. Results of the macroscopic and histological examination of the kidneys and presence of parasite DNA in the urine measured by real-time PCR. (+): scattered; +: mild alterations; ++: moderate alterations; +++: severe alterations; nd: not done

	Day post-exposure				
	12	19	26	33	47
Macroscopic enlargement of the kidneys (n = 10)					
Abundance	++	+	+ to +++	+ to +++	++ to +++
Prevalence (%)	10	30	100	100	100
Histological changes to the kidneys (n = 5)					
Prevalence (%)	100	100	100[a]	100	100
Interstitium					
Proliferation of haematopoietic tissue	+	+	+	+	+
Infiltration	+	++	+	+++	+
Necrosis		+++	+++	+++	++
Haemorrhage			+++	+++	+++
Parasites with daughter cells	+	++	+++	+++	+++
Degenerating parasites					+
Vessels					
Hypertrophy of endothelial cells		+			
Infiltration with inflammatory cells in vessel wall		+	++	++	+
Attachment of inflammatory cells		++	+++	+++	++
Parasites penetrating vessel wall			+	+	+
Necrosis of vessel wall			+	+++	+++
Thrombi			+++	+++	++
Parasites with daughter cells in lumen	+	+	++	+++	+++
Degenerating parasites					++
Tubules					
Tubulonephrosis			+	+	+
Intraluminal stages of parasites	(+)	(+)	(+)	(+)	(+)
Real-time PCR	nd	neg	pos	pos	pos

[a]n = 6

DISCUSSION

The pathogenesis of *Tetracapsuloides bryosalmonae* infection and development of pathologic lesions in the kidney with respect to water temperature are only partly understood. Further, it is not known how renal pathology relates to parasite proliferation and localization in the kidney.

The results from the present study show that fish infected with *Tetracapsuloides bryosalmonae* develop a severe proliferative and granulomatous nephritis. Congruent findings have been reported in previous studies

on fish that were kept at an elevated water temperature and showed high mortalities (Kent & Hedrick 1985, Clifton-Hadley et al. 1987), comparable to the 18°C group of the present investigation. However, the kidney pathological response at lower, more physiological temperatures, when PKD-related mortalities remain low, has not been studied so far. Our results indicate a proliferative and granulomatous nephritis does not only develop at high but also at low water temperature, as the type of renal lesion was found to be similar at the 2 temperatures. However, the severity of the lesions and their extent in the kidney tissue were markedly different. Additionally, severe vascular lesions observed at 18°C, such as vascular thrombosis and rupture, were not seen in the 12°C group.

The question arising from the findings on temperature-related differences in the intensity of renal pathology is how parasite development in the kidney responds to the temperature difference. The key findings from the comparative examination of the intrarenal fate of the parasite at 12°C and 18°C are as follows: (1) The number of parasites was significantly higher at 18°C than at 12°C. After initial infection the fish were kept in parasite-free tap water in the laboratory. It is therefore not likely that the differences in parasite loads between the 2 temperature groups resulted from parasite uptake from the environment. It is rather suggested, that the differences resulted from a temperature dependent alteration of the parasite's proliferation kinetics in the host. (2) The distribution of parasites in the renal compartments and the time course and onset of parasite degeneration were temperature independent. (3) The appearance of intraluminal parasites in the tubuli was associated with the detection of DNA in the urine. The time point of parasite DNA excretion was also temperature independent.

Because of possible changes in antigenic epitopes during parasite development, the use of ISH was included in the study as well as antigen labelling by IHC. Significant differences were found between ISH and IHC results, however these differences were found equally in both water temperature groups. Differences in the counted number of parasites by ISH and IHC could also be due to the fact that ISH identified parasites that were phagocytosed or degenerated and did not express antigens recognizable by IHC.

In conclusion, the results of this study indicate that both, proliferation of *Tetracapsuloides bryosalmonae* in a fish host and the renal pathological response to this infection are temperature dependent. These findings suggest that the increased fish mortality at 18°C is related to the increased rate of parasite proliferation and kidney dysfunction, such as impaired osmoregulation and a reduction of haematopoietic capability and haemorrhage (Roberts & Rodger 2001, Reimschuessel & Ferguson 2006). These changes may turn fatal at the higher water temperature, when the fish is confronted with increased needs of renal water excretion and of blood oxygen transport. The question of whether the advanced pathology at the higher water temperature is due to a direct effect of the increased parasite load on the renal tissue or whether it is due to factors related to the fish immunity, cannot be answered from these findings. As fish are poikilothermic animals their immune system is temperature dependent (Le Morvan et al. 1997, Köllner & Kotterba 2002, Nikoskelainen et al. 2004) so that the advanced pathological reaction especially in the interstitial tissue of the kidney might be due to an enforced immune reaction of the fish. Furthermore, while the present study focused on temperature regimes associated with significant differences in PKD manifestation to obtain insight into pathogenic processes in *T. bryosalmonae*-infected fish, the effect of smaller differences in water temperature on host-pathogen interaction remains to be investigated.

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