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Kidney pathology and parasite intensity in rainbow trout *Oncorhynchus mykiss* surviving proliferative kidney disease: time course and influence of temperature

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ABSTRACT: Proliferative kidney disease (PKD) is an endoparasitic disease of salmonids caused by the myxozoan parasite *Tetracapsuloides bryosalmonae.* We recently described the development of the disease from initial infection until manifestation of clinical disease signs in rainbow trout held at 2 water temperatures, 12 and 18°C. The aim of the present study is to investigate whether (1) infected fish surviving the clinical phase would recover from renal pathological changes, (2) whether they would be able to reduce the parasite load in the kidneys, and (3) whether water temperatures would influence renal recovery and parasite clearance. At 18°C, fish showed a gradual recovery of normal kidney morphology which was associated with a decline in parasite numbers and infection prevalence. Fish kept at 12°C initially showed an enhancement of kidney lesions before recovery of normal kidney morphology took place. The decrease in renal parasite load was retarded compared to 18°C. The results from the present study provide evidence that rainbow trout surviving the clinical phase of PKD are able to (1) fully restore renal structure, and (2) significantly reduce renal parasite loads, although 100% clearance was not achieved within the experimental period of this study. Water temperature influences the rate but not the outcome of the recovery process.

KEY WORDS: *Tetracapsuloides bryosalmonae* · Proliferative kidney disease · Temperature · Post-clinical phase · Histopathology · Parasite numbers

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INTRODUCTION

Proliferative kidney disease (PKD) is a parasitic infection of salmonids caused by *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) (Hedrick et al. 1993, Canning et al. 2000, Okamura et al. 2001). The parasite life-cycle, as currently understood, includes 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). *T. bryosalmonae* infects fish through skin and gills (Feist et al. 2001, Longshaw et al. 2002) and after invasion is distributed systemically via the blood stream. The main target organ is the kidney (Kent & Hedrick 1985) where *T. bryosalmonae* undergoes multiplication and differentiation from extrasporogonic to sporogonic stages (Kent & Hedrick 1985). Infected salmonids can excrete spores via the urine (Kent & Hedrick 1985, Morris et al. 2002, Hedrick et al. 2004, Bettge et al. 2009a,b) and transmission of the parasite from fish to bryozoans via excreted spores has been shown for brown trout *Salmo trutta* and brook trout *Salvelinus fontinalis* (Morris & Adams 2006, Grabner & ElMatbouli 2008).

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Bettge et al. (2009a,b) characterized parasite proliferation and kidney pathology in the fish host, from initial infection to the clinical phase of the disease. In rainbow trout *Oncorhynchus mykiss* reared at 18°C, the concentration of parasites and parasite DNA in the kidney increased exponentially from initial infection until it reached a plateau level at approximately 26 d post exposure (p.e.). This plateau was maintained until the end of the experiment at Day 47 p.e. In parallel to the increase of renal parasite load, renal swelling and anemia developed (Bettge et al. 2009a). Histologically, this was accompanied by a proliferative and granulomatous nephritis and a necrotizing vasculitis with thrombus formation (Bettge et al. 2009a). Furthermore, cumulative mortality among infected trout kept at 18°C steadily increased during the infection and reached 77% at Day 47 p.e.

The severity of clinical signs and lesions as well as the associated mortalities of *Tetracapsuloides bryosalmonae*-infected fish appeared to be temperature dependent (Bettge et al. 2009a). Specifically, rainbow trout infected with *T. bryosalmonae* and kept at 12°C water temperature developed less severe clinical disease signs and less severe renal pathological changes than fish kept at 18°C (Bettge et al. 2009b). In addition, cumulative mortality did not surpass 6% within the 47 d experimental period, in contrast to the 77% mortality of trout reared at 18°C. Interestingly, while the rate of increase of parasite DNA was clearly retarded at the lower water temperature when compared to that of trout kept at 18°C, maximum concentration of parasite DNA in the kidneys of 12°C trout was only moderately lower (Bettge et al. 2009b).

In trout reared at 18°C, the intensity of clinical disease signs, the severity of renal histological lesions as well as parasite intensity had shown no further enhancement from Day 26 onwards until the end of the experiment at 47 d p.e., while cumulative mortality was still increasing. This leads to the question of whether the plateau phase of the clinical and pathological parameters indicates a turning point in the disease, and whether such recovery would be accompanied by parasite clearance from the host. The available literature suggests that recovery from the kidney lesions is possible (Ferguson 1981, Kent & Hedrick 1985, Clifton-Hadley et al. 1987, Chilmonczyk et al. 2002, Morris et al. 2005). However, a systematic study on the temporal sequence of tissue repair and/or on the fate of the parasite in the host kidney has not been published to date. In addition, although water temperature has a major influence on the development of clinical disease (Bettge et al. 2009a,b), its possible role in the recovery process has not yet been studied.

The aim of the present study was to investigate mortality, renal histopathology and renal parasite load in rainbow trout infected with *Tetracapsuloides bryosalmonae*, starting from the clinical phase of PKD onwards, with regards to survival and the progress of renal histopathology, parasite load and distribution in the kidneys. In order to evaluate the role of water temperature on the recovery process, fish were kept at both 18 and 12°C.

MATERIALS AND METHODS

Experimental exposure and sampling

Fish used for the present study originated from a former experiment in which 420 naive 0+ rainbow trout *Oncorhynchus mykiss* were infected by exposing the fish to water from a *Tetracapsuloides bryosalmonae*-carrying river (Bettge et al. 2009a,b). After 5 d of exposure, infection of the exposed fish had reached 100% prevalence (n = 20), as demonstrated by means of real-time PCR on *T. bryosalmonae* DNA in the fish kidney. Thereafter, 400 infected fish were transferred into the laboratory and kept in *T. bryosalmonae*-free tap water. The fish were subdivided into 2 groups and kept at 12 and 18°C (n ≈ 200 per temperature). At Day 47 post exposure (p.e.), fish kept at 18°C had reached a plateau phase of clinical disease signs, renal parasite concentrations and renal histopathology (Bettge et al. 2009a) (see Table 1 for summary of lesions, see also Figs. 1 & 4). For the present study, fish surviving this clinical phase of PKD (18°C: 42 fish; 12°C: 151 fish) were maintained at the respective temperatures, and sampled to examine the further progress of the disease. An initial sampling of 10 animals was made at Day 47 p.e. The remaining 32 fish in the 18°C group were maintained for another 84 d (until Day 131 p.e.) and the remaining 141 fish in the 12°C group for 154 d (until Day 201 p.e.). The initial sampling of 10 fish from both groups (12 and 18°C) at 47 d p.e., was followed by 3 further samplings of 4 to 10 fish in the 18°C group (75, 103, 131 d p.e.), and 5 samplings of 10 fish in the 12°C group (75, 103, 131, 159 and 201 d p.e.) (see Table 1).

Fish were kept as described by Bettge et al. (2009a). Mortalities were recorded daily, and fish dying between the sampling days were necropsied and investigated parasitologically as described below.

Sampled fish were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories). Length (L) and weight (W) were recorded and a condition factor calculated ($WL^{-3} \times 100$). A standard necropsy was performed on all fish.

Five fish per sample (if available) were examined for the presence of parasites other than *Tetracapsuloides bryosalmonae* by microscopical evaluation of fresh mounts from the skin, the gills and the intestinal content. Fish were dissected and assessed for macroscopic changes typical of a PKD infection, such as darkening of skin, exophthalmia, pale gills, kidney swelling or ascites. The degree of kidney swelling was graded as 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe). The kidneys were removed and cut along the longitudinal axis into 2 equal parts. One half was frozen in liquid nitrogen for PCR analysis, while the other half was fixed in 10% buffered formalin for histopathology, immunohistochemical staining (IHC) and *in situ* hybridisation (ISH).

To analyze fish urine for the presence of *Tetracapsuloides bryosalmonae,* 5 fish of each group were held for 30 min before euthanasia in 2 l tanks containing tap water. It was assumed that salmonid fish release urine approximately every 30 min (Curtis & Wood 1991). After removing the fish, the water was filtered through a 4–12 µm filter paper mesh (Schleicher & Schuell MicroScience). The filters were stored at −20°C until further analysis.

Histopathology, IHC and ISH

Formalin-fixed kidney samples were paraffin-embedded and routinely processed for histological examination. Three consecutive sections of 3 µm thickness were prepared for histopathology (haematoxylin-eosin stain, H&E), IHC using a monoclonal anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics) (Adams et al. 1992) and ISH for *T. bryosalmonae* using digoxigenin-labeled probes according to Longshaw et al. (2002) and Bettge et al. (2009a). Histopathological lesions were graded 0 to 6 as for the degree of kidney swelling. Developing nephrons (nephron neogenesis) were classified according to Watanabe et al. (2009), whereby relatively small condensed basophilic cell clusters consisting of both mesenchymal condensate and nephrogenic bodies were considered as developing nephrons.

Six microscopic fields (160×) per IHC and ISH treated slide were randomly selected and the number of parasites per field counted at different organ locations (renal interstitial tissue, vessels, tubules). For each time point the mean value for all examined fish per temperature group was calculated.

Real-time PCR for detection of parasite DNA in kidney tissue and fish urine

Total DNA of rainbow trout kidneys was extracted from frozen kidney tissue with DNAzol (Lucerna) according to the manufacturer's protocol. For extracting genomic DNA from the fish urine the uppermost layer of the stored filters was removed with a scalpel blade and the total DNA extracted from this material using DNAzol. Real-time PCR was performed according to the method described by Bettge et al. (2009b).

Statistical analysis

The cumulative mortality was defined as the number of fish that died during the experiment. Significant differences between the 2 groups were tested using the χ^2 test at a $p \leq 0.05$ significance level. The condition indexes of the fish were analysed using Student's *t*-test. The amounts of parasite DNA as determined by real-time PCR were tested for normal distribution with the Skewness, Kurtosis and Omnibus Normality tests, and then compared and tested for significant differences using a 2-way ANOVA with the Tukey Kramer test as follow-up. The results of the IHC and ISH counts from each group were compared at every sampling point. As the values were not normally distributed, significant differences were tested using the Mann-Whitney *U* or Wilcoxon Rank-Sum test with a $p \leq 0.05$ significance level. The results were also assessed for differences between the quantitative real-time PCR, IHC and ISH counting methods using the Spearman rank correlation. For all statistical tests, NCSS 2001 (Hintze 2006) was used.

RESULTS

Mortality

After the start of the present experiment (47 d p.e.), a mortality of 11 fish occurred during the first 15 d in the 18°C group (n = 32), with no further mortalities thereafter. In the 12°C group (n = 141) the mortality

was significantly lower (3 fish) and was restricted to the first week (47–54 d p.e.) of the experiment.

Condition index

In both groups (18 and 12°C), condition indexes of individual fish varied from 0.9 to 1.4 throughout the experiment. No significant differences were observed, neither between sampling points nor temperature groups (data not shown).

Infection prevalence

In the 18°C group, the prevalence of animals positive for *Tetracapsuloides bryosalmonae* DNA, as determined by real-time PCR, dropped from 100% at 47 d p.e. to 25% at the end of the experiment (131 d p.e.). In the 12°C group, prevalence of positive animals dropped from 100% at 47 d p.e. to 20% at Day 131 p.e., and to 11% at the end of the experiment (Day 201 p.e.).

Concentrations of parasites and parasite DNA in fish kidney and urine

18°C group. Quantitative PCR showed a gradual decline in parasite DNA copy numbers per gram of kidney tissue over time (Fig. 1). This decline was significant between 47 and 75 d p.e.; 2-way ANOVA showed that the copy numbers of Days 75 and 103 both differed significantly from those of Day 47. At 131 d p.e., in the only positive fish, 2.57×10^3 copy numbers were found. The decline in parasite DNA was exponential ($r^2 = 0.894$).

Using IHC and ISH, the number of *Tetracapsuloides bryosalmonae* in the kidneys decreased significantly between 47 and 75 d p.e. (Fig. 2a). From 103 d p.e. onwards no parasites were detected, apart from a single parasite detected by ISH in the interstitium of 1 fish at 131 d p.e. At 47 d p.e., the infection was most intense in the kidney interstitium while in the other organ compartments only a limited number of parasites were present (Figs. 2a & 3, Table A1 in Appendix 1). Parasites were detectable by IHC in the renal interstitium until 75 d p.e., while in kidney blood vessels and renal tubules, no parasites were found after 47 d p.e. (Fig. 2a, Table A1). The results from ISH and IHC were comparable with respect to the time-dependent changes of parasite numbers in the 3 renal compartments (Fig. 2a). At 47 d p.e., there were significantly more parasites detectable in the renal interstitium by ISH compared to IHC (Fig. 2a). In the urine, parasite DNA was detected only at 47 d p.e.

12°C group. Copy numbers of parasite DNA gradually declined with time, from 4.82×10^5 (mean value from 9 to 10 animals) at Day 47 p.e. to 5.74×10^2 in the single positive fish found in the last sampling at 201 d p.e. (Fig. 1). The parasite DNA decline in the 12°C group followed an exponential curve ($r^2 = 0.879$) and seemed to be slower than in the 18°C group. Two-way ANOVA showed a significant difference between the copy numbers of Day 159 and those of Days 47 and 75.

Between-group differences in renal parasite DNA concentrations between 18 and 12°C were non-significant at all sampling time points (Fig. 1) and 2-way ANOVA revealed no interaction between both treatments.

By counting the parasites in the kidney using IHC and ISH, the highest parasite numbers were again found in the interstitium, whereas renal blood vessels and tubules displayed only low numbers of parasites (Fig. 2b). The number of parasites showed no significant change between 47 and 103 d p.e., and after 103 d p.e. parasites were no longer detected in the histological sections. In comparison to the 18°C group, the 12°C group showed significantly lower parasite numbers in the interstitium at 47 d p.e. (Fig. 3), but not at any later time point (Table A1). Similarly to the 18°C group, parasite DNA in the urine of 12°C fish was detected only at 47 d p.e.

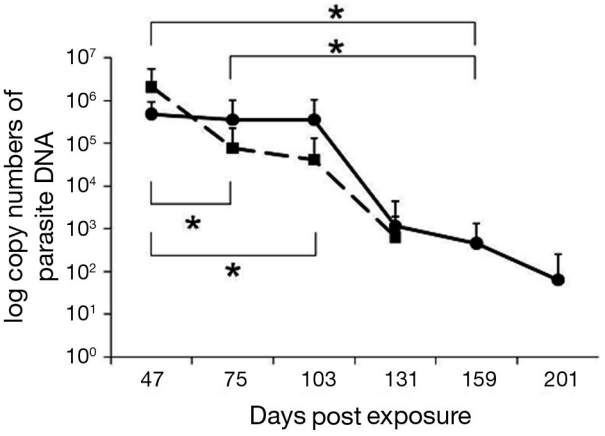

Fig. 1. Intensity of infection as estimated from PCR detection of *Tetracapsuloides bryosalmonae* DNA in kidneys of infected rainbow trout *Oncorhynchus mykiss*, showing the mean (+SD) copy numbers of parasite DNA per 2 µg of total DNA. Dashed line: 18°C group; solid line: 12°C group. Asterisks indicate significant differences between samples ($p \leq 0.05$) in the 18°C group (below) and in the 12°C group (above)

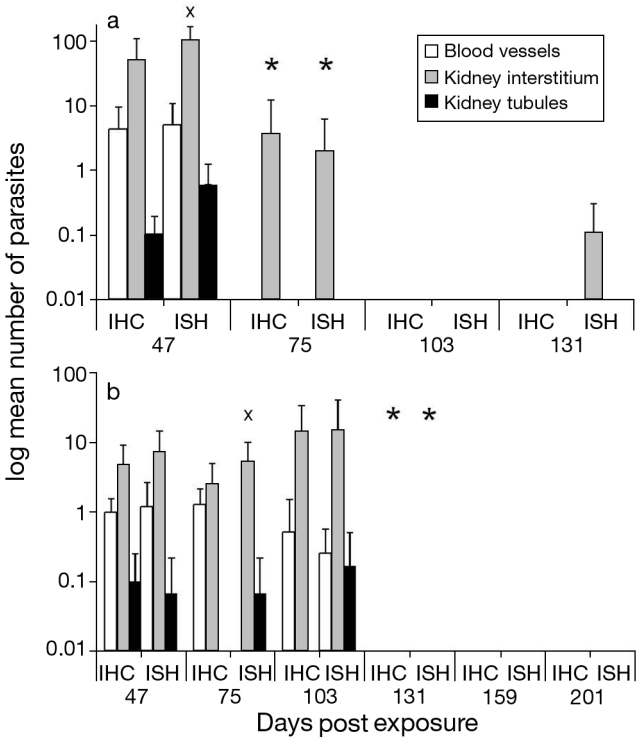

Fig. 2. Numbers of *Tetracapsuloides bryosalmonae* (logarithmic mean + SD) in 3 kidney compartments of rainbow trout *Oncorhynchus mykiss* held at (a) 18ºC and (b) 12ºC, estimated using immunohistochemical staining (IHC) and *in situ* hybridization (ISH). Significant differences ($p \leq 0.05$) are shown between consecutive samples (*) and the 2 methods used (x)

Renal pathology

18°C group. The prevalence of fish showing kidney swelling dropped from 100% at 47 d p.e. to 60% at 75 d p.e., while at 103 and 131 d p.e., kidneys of all fish appeared macroscopically normal (Table 1). Histologically, the extent of severity of renal proliferative and degenerative changes such as interstitial proliferation and necrosis, as well as fibrosis of interstitial tissue, decreased from 47 to 75 d p.e. (Figs. 4a,b & 5a, Table A2 in Appendix 1). Degenerating parasites, characterized by hypereosinophilia and fragmentation of parasite cells and nuclei, were present in the interstitium at 47 and 75 d p.e. From 103 d p.e. onwards, no histopathological lesions were detectable in the kidneys. At 103 d p.e., some specimens examined showed an increase in the number of newly formed renal tubules, characterized by basophilic cell clusters with numerous mitotic figures, indicating nephron neogenesis (Fig. 5c).

12°C group. Prevalence of fish displaying macroscopic signs of PKD decreased continuously over the experimental period and at the last 2 sampling points no fish showed clinical symptoms (Table 1). The extent of the macroscopic lesions increased until 103 d p.e. (severe kidney swelling in several animals) and subsequently decreased. Only mild lesions were observed in all cases at 131 d p.e. and none at later sampling points.

Table 1. Presence and effects of *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss* held at 18 and 12°C, evaluated by means of macroscopic evaluation, real-time PCR, immunohistochemical staining (IHC), *in situ* hybridization (ISH) and histological examination of kidneys. IP: Infection prevalence. Severity of macroscopic changes is given as the mean value of kidney swelling indices of the individual fishes examined during necropsy and (in parentheses) lowest and highest values per sampling. Severity of the histological changes is given as mean value calculated from the histopathological indices of the individual fishes. Detailed data are shown in Tables A1 & A2

	Day 47		Day 75		Day 103		Day 131		Day 159	Day 201
	18°C	12°C	18°C	12°C	18°C	12°C	18°C	12°C	12°C	12°C
Macroscopic swelling of the kidneys										
N	10	10	5	10	5	10	5	10	10	10
Percentage (%)	100	90	60	60	0	60	0	10	0	0
Mean severity	5 (4–6)	3 (0–4)	3 (0–4)	4 (0–6)	0 (0–0)	4 (0–6)	0 (0–0)	0 (0–2)	0 (0–0)	0 (0–0)
Real-time PCR on *T. bryosalmonae* DNA										
N	10	10	5	10	5	10	4	10	9	9
IP (%)	100	100	100	100	100	90	25	20	44.4	11.1
IHC of *T. bryosalmonae*										
N	5	5	5	5	2	4	3	5	5	5
IP (%)	80	100	20	100	0	75	0	0	0	0
ISH of *T. bryosalmonae*										
N	5	5	5	5	2	4	3	5	5	5
IP (%)	100	100	40	100	0	75	33	0	0	0
Histological changes to the kidneys										
N	5	5	5	5	2	5	3	5	5	5
Percentage (%)	100	100	80	100	100	100	0	60	100	0
Mean severity	2.8	0.7	1	1.1	0.2	2.8	0	0.2	0.5	0

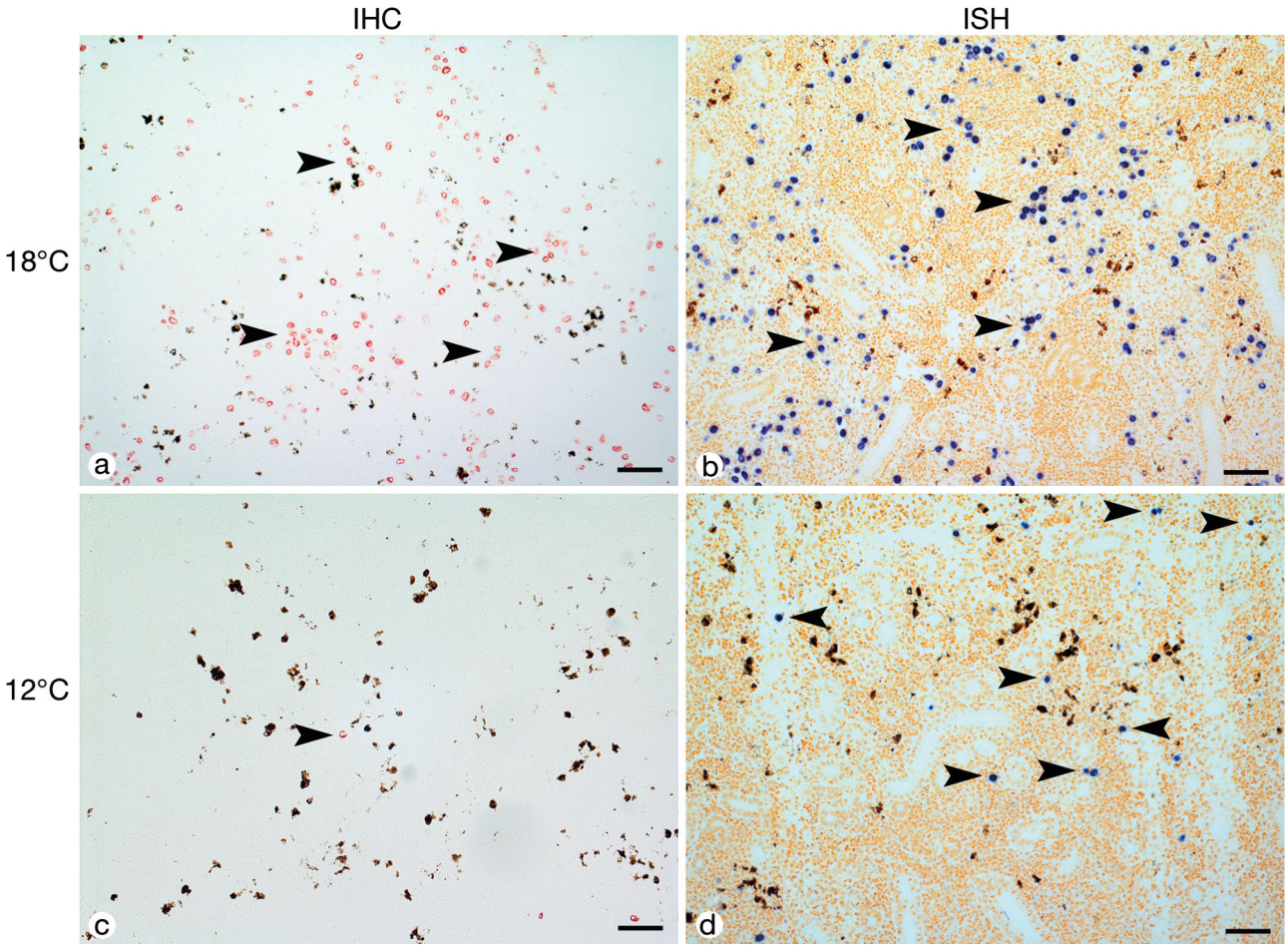

Fig. 3. Presence of *Tetracapsuloides bryosalmonae* in the kidney interstitium of rainbow trout *Oncorhynchus mykiss* at 47 d post exposure, shown by immunohistochemical staining (IHC) and *in situ* hybridization (ISH). (a) IHC and (b) ISH show high numbers of parasites in fish kept at 18°C. (c) IHC and (d) ISH show small numbers of parasites in fish kept at 12°C. Black arrowheads indicate single parasites. Scale bars = 50 µm

The prevalence of animals with histological lesions remained high until 159 d p.e. but at 201 d p.e. lesions were no longer detected. Histological kidney lesions increased slightly in the beginning of the experiment (Fig. 4c), reaching a peak at 103 d p.e. At this time point, a marked proliferative and granulomatous nephritis associated with necrotizing vasculitis, vascular thrombosis, scattered hemorrhage and multiple areas of necrosis were present in most of the affected animals (Fig. 5b,d, Table A2). In contrast, at 131 and 159 d p.e., only mild lesions were visible. Intralesional parasites were present both in the interstitium and in renal blood vessels until 103 d p.e. From the start of the experiment, interstitial parasites showed signs of degeneration and were surrounded by macrophages.

At 131 and 159 d p.e. proliferation and infiltration in the interstitium were replaced by fibrosis. Increased numbers of developing nephrons (nephron neogenesis) were detectable from 103 d p.e. onwards (Fig. 5e, Table A2). At the last sampling point (201 d p.e.), normal kidney structure was restored in all investigated animals and no pathological changes were detected.

Comparison between the different methods

To evaluate whether changes of parasite DNA copy numbers measured by real-time PCR correlate with changes in parasite numbers as seen on IHC and ISH sections, the PCR samples were compared to results for the same individuals obtained using IHC and ISH. For fish held at 12°C, there were linear correlations between PCR and IHC results, with a correlation coefficient of $r^2 = 0.7167$, and between PCR and ISH

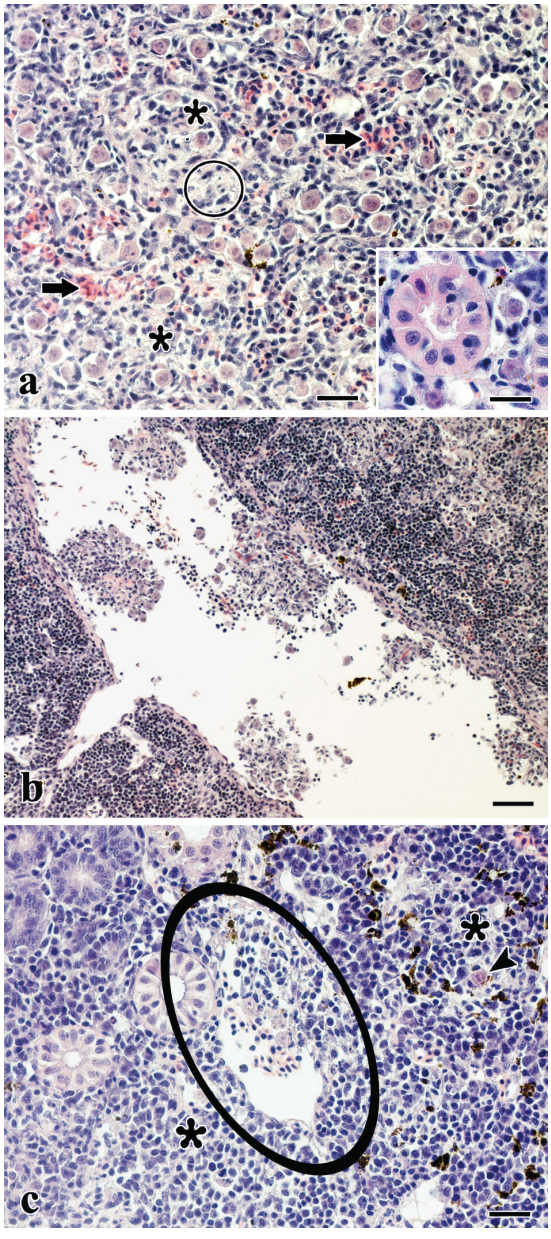

Fig. 4. *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss*, showing histological changes and parasite localization in fish held at (a,b) 18°C and (c) 12°C at 47 d post exposure. (a) The kidney interstitium is severely expanded mainly as a result of infiltration by macrophages (*), with the presence of necrotic cell debris (circle), hemorrhages (arrows) and parasites. Inset: Parasites extruding from the epithelium into the lumen of a renal tubule. (b) Renal blood vessel with multiple thrombi consisting of inflammatory cells (macrophages, lymphocytes and plasma cells; often degenerated), cell debris and parasites. (c) Renal blood vessel with thrombus consisting of macrophages, lymphocytes, plasma cells and parasites (inside circle). The renal interstitial tissue is proliferated and contains scattered *T. bryosalmonae* (black arrowhead) and mild infiltration with macrophages (*) in the interstitium. H&E staining. Scale bars = (a,inset,c) 25 µm; (b) 50 µm

results, with r^2 = 0.9283. In contrast, in fish with immunohistochemically intense infections (>50 parasites per microscopic slide, as seen in the 18°C group), no correlation between PCR results and parasite counts was found (r^2 = 0.0535). Similarly, there was no correlation between PCR results and those obtained from ISH-stained sections in the 18°C group (r^2 = 0.1453).

DISCUSSION

The present study examined the progress of mortality, parasite infection and renal pathology of *Tetracapsuloides bryosalmonae*-infected rainbow trout after the clinical disease stage of PKD. Bettge et al. (2009a,b) showed that, for trout kept at 18°C, no further enhancement of clinical disease signs, no increase in the severity of histopathological lesions and no further elevation of renal parasite loads occurred between 21 and 47 d p.e. Apparently, the disease severity had reached a kind of plateau. In contrast, mortality continued to increase from 21 to 47 d p.e., reaching 77% on Day 47. However, in the phase after 47 d p.e., which is investigated in the present study, mortality in the 18°C group was low, increasing by only 11 fish. In parallel, disease severity decreased; in particular, we observed (1) a disappearance of clinical disease signs, (2) a full recovery of normal renal structure, and (3) a significant reduction of the prevalence of infected fish as well as a significant decline of renal parasite loads in infected individuals. The situation was different in the 12°C group (Fig. 6). Prevalence of infected fish was 100%, as in the 18°C group. However, the increase of parasite DNA in the kidneys occurred more slowly than at 18°C and the maximum level was lower. Cumulative mortality remained low until 47 d p.e., not surpassing 6%. Similarly, clinical disease signs as well as renal histopathology were moderate (Bettge et al. 2009b). After 47 d p.e., the 12°C group displayed a pattern somewhat different from the 18°C group. Cumulative mortality remained low, reaching a maximum of 8%.The prevalence of *T. bryosalmonae*-infected fish as well as renal concentrations of parasite DNA continuously decreased, as in the 18°C group, but the decrease was slower at 12°C. In contrast to the 18°C group, renal histopathology showed an initial increase in severity until Day 103 p.e. Afterwards, recovery of normal renal morphology was observed. In summary, these findings suggest that individuals surviving the PKD phase associated with maximum clinical signs and maximum mortality are able to

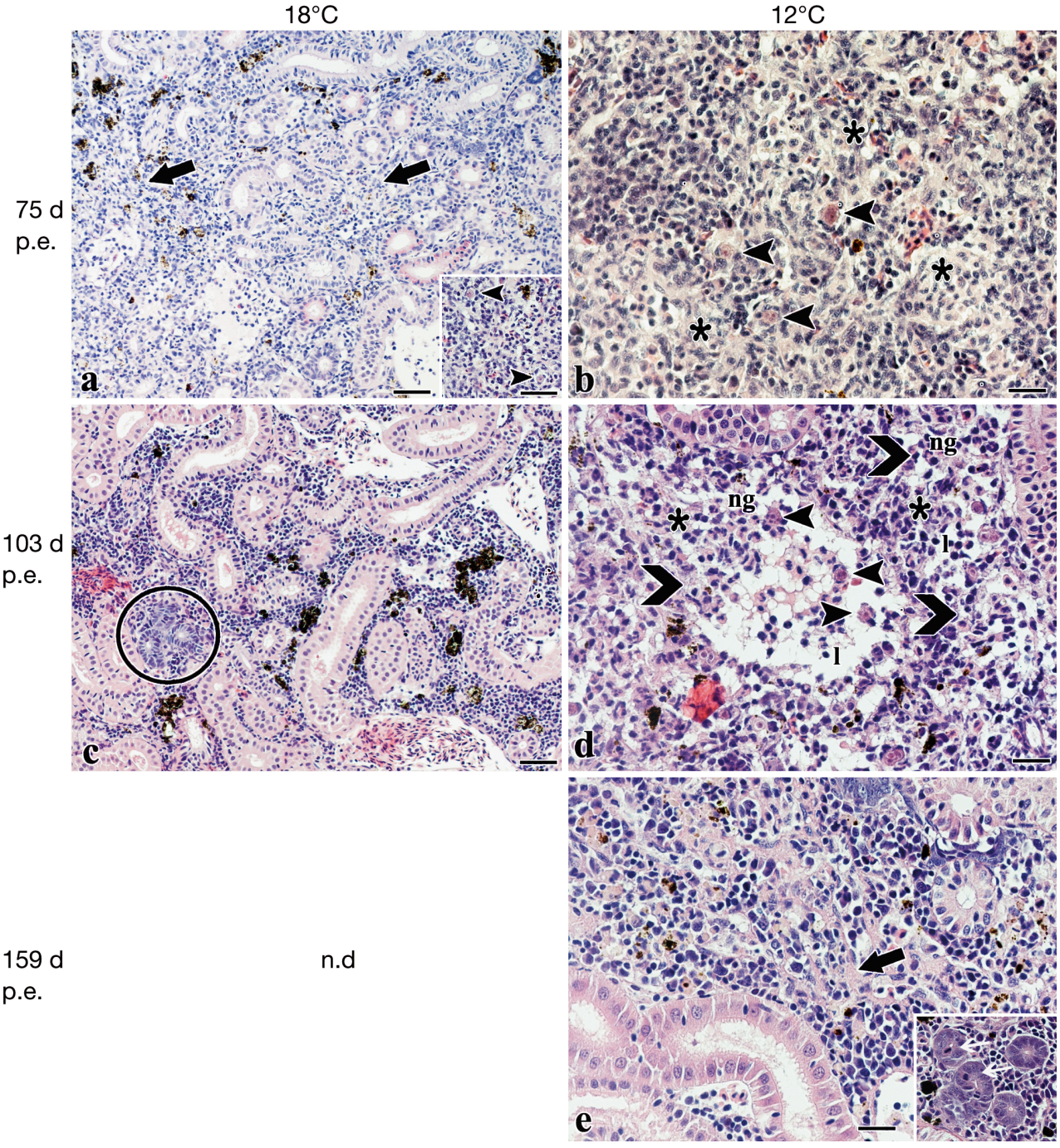

Fig. 5. *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss*, showing histological changes and distribution of parasites in fish held at 18°C (left) and at 12°C (right) at (a,b) 75 d, (c,d) 103 d and (e) 159 d post exposure (p.e.). No fish remained in the 18°C group at 159 d p.e. (n.d.). (a) Small areas with interstitial fibrosis (black arrows); inset shows scattered parasites (arrowheads). (b) Interstitium is expanded as a result of infiltration, mainly by macrophages (*); moderate numbers of parasites (black arrowheads) are also present. (c) Interstitial tissue fully restored, with numerous developing nephrons, characterized by a basophilic cytoplasm and a high nucleus/cytoplasm ratio (inside black circle). (d) Large areas of necrosis (black open arrows) in renal interstitium with fibrin exudation and infiltration with macrophages (*), lymphocytes (l), and neutrophilic granulocytes (nG). Parasites are surrounded by inflammatory cells (arrowheads). (e) Moderate amount of interstitial fibrosis (black arrow); inset shows numerous developing nephrons, characterized by a basophilic cytoplasm and a high nucleus/cytoplasm ratio, and the presence of mitotic figures (white arrows). H&E staining. Scale bars = (a,inset,c) 50 µm, (b,d,e,inset) 25 µm

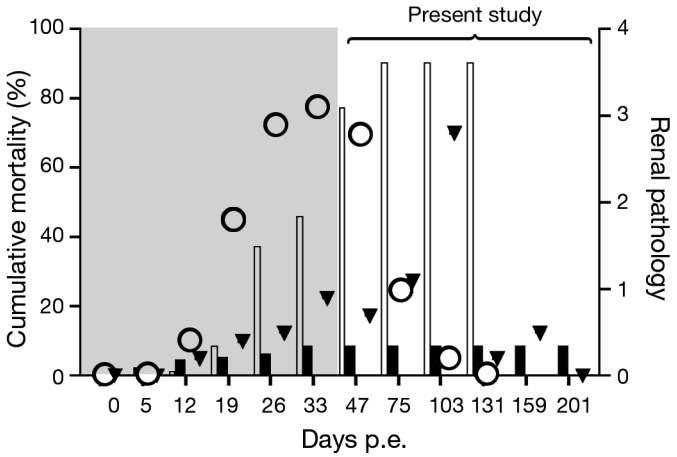

Fig. 6. Development of cumulative mortality (left *y*-axis) and of severity of renal pathology (right *y*-axis) in rainbow trout *Oncorhynchus mykiss* infected by *Tetracapsuloides bryosalmonae*, from initial infection until peak of clinical disease at 47 d post exposure (p.e.) (grey shaded area; see Bettge et al. 2009a) and from Day 47 until the end of the present study 201 d p.e. Cumulative mortality is given for fish reared at 18°C (white bars), and 12°C (black bars). Histological changes were semi-quantitatively graded on a scale from 0 to 6; the data shown represent mean values for 5 fish of scores based on analysis of the renal interstitium, blood vessels and tubules. Histopathological scores are shown for the 18°C (○) and 12°C (▼) treatments

recover, independently of the water temperature. Furthermore, our results indicate a clearance of the parasite from the kidney at high and low water temperatures. Whereas by IHC and ISH almost no parasites were detectable beyond 75 d p.e. at 18°C and 103 d p.e. at 12°C, low levels of parasite DNA were still detectable in single animals until the end of the experiment. These results can be explained by methodological differences. Whereas only one section through the kidney tissue can be examined by IHC or ISH, real-time PCR enables investigation of the whole kidney sample. As long as the parasite numbers were below 50 per microscopic field, a good correlation between PCR and IHC/ISH was found, providing evidence to support the reliability of the PCR quantification method. The poor correlation in the 18°C group can be explained by the small number of sections examined and the presence of numerous and clustering parasites.

Whether the DNA found at the end of the experiments in single fish of both groups reflects functional parasites, which could cause a new disease outbreak under suitable conditions, has still to be investigated. Both techniques (direct counting and real-time PCR) indicate an accelerated elimination of the parasites at the higher water temperature. This may be due to an influence of temperature on the immune system of the fish, probably affecting the elimination of the parasites by the host (for reviews see Bowden et al. 2007, Bowden 2008, Magnadóttir 2006).

The severity of clinical signs and the associated mortality in *Tetracapsuloides bryosalmonae*-infected fish were temperature dependent (Bettge et al. 2009a). This has also been shown for other myxozoan parasites, such as *Enteromyxum* spp. and *Myxobolus cerebralis* (Yanagida et al. 2006, Murcia et al. 2011). However, whereas histopathological changes also increased with higher water temperature in *Myxobolus cerebralis*-infected cutthroat trout, in the present study only the temporal development of histopathology was accelerated at a higher water temperature. In the present study, the severity of lesions was less influenced by water temperature.

Several studies have shown a regression of renal lesions in rainbow trout surviving the clinical phase of PKD (Ferguson 1981, Kent & Hedrick 1985, Clifton-Hadley et al. 1987). Fish are able to regenerate renal tissue by repopulation of injured nephrons and, unlike mammals, by the de novo production of nephrons. Renal regeneration by nephron neogenesis has been reported in catfish, rainbow trout, tomcod, zebrafish, tilapia, aglomerular toadfish and medaka (Reimschuessel et al. 1990, 1996, Reimschuessel 2001, Salice et al. 2001, Watanabe et al. 2009, Diep et al. 2011) These nephrons arise from basophilic cell clusters in the interstitium and progress through the normal stages of nephron differentiation. The nephrogenic zone was identified as the region where cell proliferation and mesenchymal cell aggregation is enhanced, and is considered to contain renal stem cell-like mesenchymal cells (Elger et al. 2003). Chilmonczyk et al. (2002) and Morris et al. (2005) reported that a small percentage of animals surviving the clinical phase of PKD still show histopathological lesions up to 27 or 40 wk after infection. In Bettge et al. (2009a) and the present study, in which trout were kept for 28 wk from initial infection and for 22 wk beyond the main clinical phase of the disease, full regeneration of normal renal structure was observed. This indicates that PKD-infected fish can indeed recover from lesions. Importantly, while all aforementioned studies examined kidney regeneration at water temperatures higher than 15°C, the results from the present study demonstrate that full structural regeneration of kidneys is also possible at lower water temperatures (12°C). In the natural environment, PKD outbreaks in feral and farmed fish usually occur during summer months, and the recovery process takes place

towards autumn. Thus, the 12°C experiment may well correspond to the recovery condition in free-ranging salmonids. Ferguson (1981) found that the recovery process in rainbow trout occured faster at lower water temperatures. In contrast, in zebrafish it has been shown that renal injury, repair and nephron neogenesis is enhanced with increasing water temperature (Reimschuessel & Ferguson 2006). In the present study, however, the time course of renal regeneration itself seemed to be temperature independent. However, we observed an interesting difference in the process of renal histological regeneration between the 2 temperature groups: in the 12°C group, the severity of lesions initially (from 47 to 103 d p.e.) increased to a level comparable to the intensity in 18°C fish at 47 d p.e., and only afterwards, i.e. beyond Day 103 p.e., a recovery process started. This suggests that the initiation of the recovery process may be triggered by the intensity of the host tissue response, rather than being a function of the duration of infection.

In conclusion, the present study indicates that the severity of renal pathology in PKD-infected rainbow trout is temperature independent whereas the temporal development is temperature dependent with parasite numbers possibly correlating to the time course but not to the severity of lesions (Fig. 6). Moreover, we showed that rainbow trout are able to completely recover from pathological renal lesions caused by an infection with *Tetracapsuloides bryosalmonae.* In parallel to this, clearance of parasites from the host kidney takes place. Whether the elimination of parasites from the kidney is complete, or viable parasites are still present in the recovered kidneys, remains to be elucidated.

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Table A1. Comparison of numbers of *Tetracapsuloides bryosalmonae* in kidneys of rainbow trout *Oncorhynchus mykiss* kept at 18 and 12°C, in assessments by immunohistochemical staining (IHC) and *in situ* hybridization (ISH) in 3 kidney compartments: blood vessels, interstitium and tubules. Numbers in parentheses show lowest and highest values of parasites counted in 6 microscopic fields (160×) per fish (n = 5) per sample point. Significant differences ($p \leq 0.05$) are shown between consecutive samples (⋆), the 2 methods used on the same samples (x), and the 2 temperature groups at the same sample point and using the same method (y). p.e.: post exposure

Histological technique	Day 47		Day 75		Day 103		Day 131		Day 201	Day 159
	18°C	12°C	18°C	12°C	18°C	12°C	18°C	12°C	12°C	12°C
Vessels										
IHC	4.27 (0–30)	0.97 (0–6)	$0.00^{y\star}$ (0–0)	1.27 (0–6)	0 (0–0)	0.5 (0–3)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
ISH	4.93 (0–23)	1.17 (0–9)	$0.00^{\star}$ (0–0)	0.00^{x} (0–0)	0 (0–0)	0.25 (0–2)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Interstitium										
IHC	52.00^{y} (0–200)	4.87 (0–14)	$3.80^{\star}$ (0–26)	2.53 (0–8)	0 (0–0)	14.59 (0–63)	0 (0–0)	$0.00^{\star}$ (0–0)	0 (0–0)	0 (0–0)
ISH	103.73^{xy} (1–227)	7.4 (1–30)	$2.00^{\star}$ (0–13)	5.33 (0–18)	0 (0–0)	15.17 (0–76)	0.11 (0–1)	$0.00^{\star}$ (0–0)	0 (0–0)	0 (0–0)
Tubules										
IHC	0.1 (0–1)	0.1 (0–2)	$0.00^{\star}$ (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
ISH	0.60^{x} (0–3)	0.07 (0–2)	$0.00^{\star}$ (0–0)	0.07 (0–1)	0 (0–0)	0.17 (0–2)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)

Table A2. Results of histopathological examination of the kidneys of rainbow trout *Oncorhynchus mykiss* infected by *Tetracapsuloides bryosalmonae* and held at 18 and 12°C, showing mean values between 47 and 201 d post exposure of examined animals (n = 5, if available) and (in parentheses) lowest and highest values, based on the following scoring criteria: (0) no lesions; (1) minimal lesions; (2) mild lesions; (3) mild to moderate lesions; (4) moderate lesions; (5) moderate to severe lesions; (6) severe lesions

	Day 47		Day 75		Day 103		Day 131		Day 159	Day 201
	18°C	12°C	18°C	12°C	18°C	12°C	18°C	12°C	12°C	12°C
Interstitium										
Proliferation of hematopoietic tissue	1 (0–2)	3 (2–4)	1 (0–2)	2 (0–4)	0 (0–0)	2 (2–2)	0 (0–0)	0 (0–0)	0 (0–2)	0 (0–0)
Infiltration	5 (4–6)	2 (2–4)	2 (0–5)	2 (1–4)	0 (0–0)	5 (3–6)	0 (0–0)	0 (0–2)	1 (0–2)	0 (0–0)
Necrosis	4 (2–6)	2 (0–3)	1 (0–4)	1 (0–2)	0 (0–0)	3 (2–4)	0 (0–0)	0 (0–0)	1 (0–2)	0 (0–0)
Hemorrhage	2 (0–5)	0 (0–0)	1 (0–2)	1 (0–2)	0 (0–0)	2 (0–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Fibrosis	2 (0–3)	0 (0–0)	1 (0–4)	1 (0–2)	0 (0–0)	3 (2–4)	0 (0–0)	1 (0–4)	1 (0–3)	0 (0–0)
Parasites with daughter cells	5 (1–6)	3 (2–4)	1 (0–4)	2 (0–4)	0 (0–0)	3 (0–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Degenerating parasites	2 (0–4)	1 (0–2)	1 (0–2)	1 (0–2)	0 (0–0)	2 (0–2)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Vessels										
Hypertrophy of endothelial cells	0 (0–0)	2 (2–2)	1 (0–2)	1 (0–2)	0 (0–0)	2 (0–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Attachment of inflammatory cells	4 (4–4)	4 (4–6)	2 (0–4)	2 (2–4)	0 (0–0)	4 (2–6)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Necrosis of wall of vessels	4 (3–6)	2 (0–2)	0 (0–0)	1 (0–2)	0 (0–0)	3 (0–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Thrombi	4 (4–4)	1 (0–2)	1 (0–2)	1 (0–2)	0 (0–0)	4 (2–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Parasites with daughter cells in lumen	4 (1–6)	4 (4–6)	1 (0–2)	3 (1–4)	0 (0–0)	2 (0–4)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Tubules										
Tubulonephrosis	3 (2–4)	1 (0–2)	1 (0–2)	1 (0–4)	0 (0–0)	2 (2–2)	0 (0–0)	0 (0–1)	1 (0–2)	0 (0–0)
Nephron neogenesis	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	2 (2–2)	1 (0–2)	0 (0–0)	1 (0–2)	2 (2–3)	0 (0–0)
Intraluminal stages of parasites	1 (0–2)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)

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