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Comparative study of proliferative kidney disease in grayling *Thymallus thymallus* and brown trout *Salmo trutta fario*: an exposure experiment

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ABSTRACT: Proliferative kidney disease (PKD) is an emerging disease threatening wild salmonid populations, with the myxozoan parasite *Tetracapsuloides bryosalmonae* as the causative agent. Species differences in parasite susceptibility and disease-induced mortality seem to exist. The aim of the present study was to compare incidence, pathology and mortality of PKD in grayling *Thymallus thymallus* and brown trout *Salmo trutta* under identical semi-natural conditions. Young-of-the-year grayling and brown trout, free of *T. bryosalmonae*, were jointly exposed in cage compartments in a river in the northeast of Switzerland during 3 summer months. Wild brown trout were caught by electrofishing near the cage, and PKD status was compared with that of caged animals. Cage-exposed grayling showed a PKD incidence of 1%, regardless of whether parasite infection was determined by means of real-time PCR or histopathology/immunohistochemistry. In contrast, PKD incidence of caged brown trout was 77%. This value was not significantly different to PKD prevalence of wild brown trout caught above and below the cage (60 and 91%, respectively). Mortality in grayling was significantly higher compared with that of brown trout (40 versus 23%); however, grayling mortality was not considered to be associated with PKD. Mortality of caged and infected brown trout was significantly higher than mortality of non-infected caged trout. Histopathology indicated an ongoing mostly acute or chronic active infection in brown trout, which survived until the end of exposure. The results suggest that grayling are less susceptible to infection with *T. bryosalmonae* compared with brown trout under the tested field conditions.

KEY WORDS: Proliferative kidney disease · Grayling · Brown trout · Mortality · Pathology · Temperature

INTRODUCTION

Proliferative kidney disease (PKD) causes high mortality in farmed and wild salmonids and was recently classified as an emerging disease throughout Europe (Okamura et al. 2011). The disease is caused by *Tetracapsuloides bryosalmonae*, a parasite belonging to the Malacospora (Myxozoa) (Hedrick et al. 1993, Canning et al. 2000, Okamura et al. 2001). Myxozoans were recently assigned to the phylum Cnidaria (Jiménez-Guri et al. 2007, Nesnidal et al. 2013). The complex life cycle of these parasites involves 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). The ports of entry in the fish are the skin and gills (Morris et al. 2000, Feist et al. 2001, Longshaw et al. 2002, Grabner & El-Matbouli 2010). Afterwards, the parasite is distributed systemically and reaches the main target organ,

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the kidney, via the vascular system (Kent & Hedrick 1985). In the kidney, the parasites penetrate the vessel wall, inducing necrotising vasculitis with thrombi, and differentiate from extrasporogonic stages in the renal interstitium to sporogonic stages in the lumen of renal tubuli (Kent & Hedrick 1985, Bettge et al. 2009). Mature spores are excreted by urine (Kent & Hedrick 1985, Hedrick et al. 2004, Morris & Adams 2006, Bettge et al. 2009). Surviving fish can completely regenerate and restore the normal renal morphology (Schmidt-Posthaus et al. 2012, 2013).

The mainly affected age class of fish is young-of-the-year (YOY), which develop a proliferative and granulomatous nephritis (Hedrick et al. 1993, Bettge et al. 2009). The distribution of disease and mortality rates are thought to be influenced by water temperature. This is corroborated by findings in Switzerland, where fish infected by *T. bryosalmonae* were found almost exclusively below 800 m above sea level, where water temperatures are clearly higher than those above this elevation (Wahli et al. 2008). Experiments using rainbow trout *Oncorhynchus mykiss* demonstrated increased mortality at temperatures above 15°C (Bettge et al. 2009, Okamura et al. 2011). Mortality can be as high as 85% in rainbow trout (Bettge et al. 2009), whereas in brown trout *Salmo trutta fario* under field conditions a mortality of only 15% was recorded (Schmidt-Posthaus et al. 2015). The infection has been described mainly from salmonids including brown trout, rainbow trout, grayling *Thymallus thymallus*, salmon (*Salmo salar*, *Oncorhynchus tschawytscha*) and brook trout *Salvelinus fontinalis* (Hedrick et al. 1993). However, species-specific differences seem to exist, e.g. varying effects of *T. bryosalmonae* infection were reported for wild salmonids in Danish rivers (Skovgaard & Buchmann 2012). Besides brown trout, grayling is also an important native species to Swiss rivers. As mentioned, grayling were also reported to be hosts for *T. bryosalmonae* (Feist & Bucke 1993, Hedrick et al. 1993, Grabner & El Matbouli 2008), with similar pathology as described in rainbow and brown trout (Grabner & El Matbouli 2008). Grabner & El Matbouli (2008) described severe kidney and spleen swelling, proliferation of renal interstitial tissue and many interstitial parasite stages in infected grayling.

The River Wutach is known to be PKD-positive from earlier studies (Schmidt-Posthaus et al. 2015). In former years, the population of grayling in the Wutach was stable until the early 1980s, when the population was dramatically reduced by a poisoning event. Populations did not recover despite massive stocking of fry and fingerlings from 1999 to 2007 (local fishermen's statistics 1979–1996, unpubl.). Stocking was stopped in 2007. Brown trout populations have declined severely in the River Wutach as well, resulting in establishment of a stocking program.

The question arose as to whether the declines in populations of the native species grayling and brown trout might be associated with the presence of *T. bryosalmonae*. To answer this question, knowledge on prevalence and mortality due to PKD in grayling would be essential. However, to date, such data are missing.

The aim of the present study was to: (1) investigate incidence and mortality in grayling exposed to *T. bryosalmonae* in comparison to brown trout exposed under semi-natural conditions in the River Wutach; and (2) compare the pathology associated with the infection in order to assess the potential involvement of the disease in the grayling decline.

MATERIALS AND METHODS

Study sites and fish sampling

Experiments were conducted over a period of 3 mo (21 July to 17 October 2015) in the River Wutach, which has a 4.8 km border section between southern Germany and the northeastern part of Switzerland (Fig. 1). An earlier start of the experiment was not possible because of availability and size of experimental animals, especially grayling.

Investigations were conducted in 2 separate approaches: (1) to determine species-specific differences in infection sensitivity and disease characteristics, YOY grayling and YOY brown trout were exposed to river water in a cage fixed in the River Wutach for 3 months; (2) to assess the PKD status in wild fish, brown trout were sampled by electrofishing in 2 stretches in the Wutach (field sampling). Field sampling was performed as a positive reference to evaluate differences due to cage conditions. Water temperature was measured inside and near the cage in the Wutach.

Cage experiment

The cage used for the parallel exposure of YOY brown trout *Salmo trutta fario* and grayling *Thymallus thymallus* measured 200 × 120 × 50 cm and was longitudinally divided into 2 equal compartments (Fig. 1). Both front and rear, as well as the covering

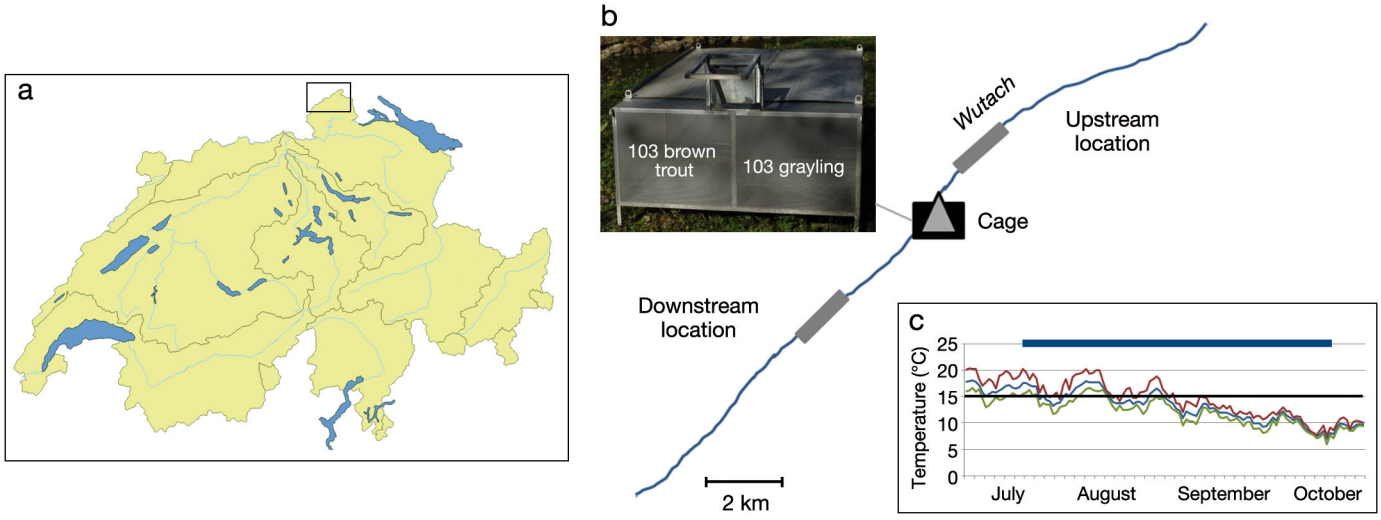

Fig. 1. (a) Map of Switzerland showing the location of the River Wutach at the border with Germany (black rectangle). (b) Schematic of the River Wutach, showing field sampling sites (grey bars), cage (black rectangle) and location of temperature logger (grey triangle) in the Wutach. The cage (see photo) was longitudinally divided into 2 equal compartments (see text for details). (c) Water temperature measured inside the cage between July and October (blue line: mean temperature curve; green: minimum values; red: maximum values). The black line indicates 15°C, which has been shown to be critical for proliferative kidney disease (PKD)-related clinical signs and mortality in trout. The bold blue line indicates the exposure time of brown trout and grayling in the river water

and the bottom plates, consisted of stainless steel mesh with 0.4 cm diameter. The 2 lateral plates and the dividing plates were made of transparent Macrolon (Röhm Schweiz). The bottom of the cage was covered with gravel. The perforation of the ground plate prevented the accumulation of mud and made cleaning of the cage unnecessary. An additional V-shaped wire mesh was placed outside of the cage in the upstream position to avoid blocking of the cage by large floating debris. This wire mesh was cleaned regularly. A total of 124 YOY grayling were acquired from a nearby farm producing grayling for stocking purposes; 124 YOY brown trout were purchased from a trout hatchery (see also Schmidt-Posthaus et al. 2015). Grayling originated from broodstock caught by net in the River Rhine. Afterwards, eggs were raised in the farm. Offspring brown trout originated also of broodstock, originally deriving from the Wutach. They were mixed on the farm. Therefore, both grayling and brown trout used in this experiment originated from various mother animals. Both fish farms were supplied by spring water. Here, water temperature remained relatively stable, never exceeding 11°C. There was no disease record in these farms.

In both farms, *Tetracapsuloides bryosalmonae* has never been found. In order to confirm the *T. bryosalmonae*-free status of the fish, a subsample of each species consisting of 10 individuals each was analysed for the presence of the parasite by real-time PCR. To exclude a potential low-level infection not detectable by real-time PCR, 11 fish of each species were kept in spring water at the grayling farm and brown trout hatchery of origin, respectively, during the entire experimental period.

The remaining 103 grayling (5.7–7.0 cm) and 103 brown trout (7.5–9.4 cm) were placed into the cage. They were fed with commercial fish food (Skretting, Aqua Brut pellets 1.0–1.5 mm) using an automatic feeding machine dispensing a continuous food supply of approximately 2% of total body weight per day. Fish were monitored daily. Moribund or dead fish were removed and necropsied immediately. Three months after the start of the exposure experiment, all remaining grayling and trout were euthanized in clove oil, tagged and immediately examined. The abdominal cavity was opened and the macroscopic aspect of the kidney was photographically documented.

Field sampling

Wild brown trout were sampled from 2 stretches of 100 m in the River Wutach, one stretch 2 to 3 km upstream of the cage experiment and one stretch 3 to 4 km downstream, before any stocking (Fig. 1). In September 2015, 10 and 11 YOY brown trout, respec-

tively, were sampled at each of the 2 sites in the Wutach by electrofishing. Because grayling are absent in the Wutach it was not possible to sample wild grayling for this study. Brown trout were euthanized in clove oil and examined as described below.

Pathology, histopathology and immunohistochemistry

Length of every dead or euthanized fish was recorded, followed by a complete necropsy. Individuals originating from the cage experiment were additionally weighed and the condition factor was calculated ($100 \times \text{weight/length}^3$; Bagenal 1978, Froese & Pauly 2016). Animals were necropsied, macroscopic changes in the inner organs were evaluated and the kidney was photographed to document gross signs. Inner organs except the kidney were removed, after which whole animals were immediately fixed in 10% buffered formalin and sent to the Centre for Fish and Wildlife Health (CFWH) for histopathological and immunohistochemical (IHC) examination. At the CFWH, kidneys were removed and routinely prepared for histology and IHC according to Schmidt-Posthaus et al. (2015). For IHC, a monoclonal anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics) was used according to Adams et al. (1992). In renal histopathology, the following criteria were judged (according to the presumed temporal development): proliferation of interstitial tissue; vascular degeneration and inflammation with mainly macrophages and lymphocytes, thrombi; interstitial necrosis, interstitial haemorrhage; interstitial inflammation with mainly macrophages; interstitial fibrosis; tubulonephrosis and tubuloneogenesis. These histopathological changes were graded as 0 (none) to 6 (severe). Presence of *T. bryosalmonae* was examined on whole histological and IHC sections and infection intensity was classified as 0 (no parasites present on whole slide), 1 (scattered parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a magnification of 200 to 400×.

Real-time PCR for detection of *T. bryosalmonae* DNA in kidney tissue

Fish negative for *T. bryosalmonae* by histology were analysed by real-time PCR as described by Schmidt-Posthaus et al. (2015). Briefly, two 20 µm sections of paraffin embedded material were deparaffinized. Real-time PCR was performed using SYBR GoTaq qPCR Master Mix (Promega, Dübendorf, Switzerland) according to the manufacturer's instructions. The primer pair PKX3F (CTA AGT ACA TAC TTC GGT AGA) and PKX4R (CCG TTA CAA CCT TGT TAG GAA), described by Kent et al. (1998), was used. A histologically positive sample from another study was used as a positive control. Water served as a negative control. A 297 bp gene sequence of the small subunit ribosomal DNA (SSU rDNA) was detected. To verify the sequence, selected PCR products were sent to Microsynth AG, Balgach, Switzerland, for sequencing. The identity of PCR products was determined by BLAST-n searching of the available sequences in the GenBank database (www.ncbi.nlm.nih.gov).

Water temperature

Water temperature was recorded every 2 h by temperature loggers located inside the cage and in the Wutach near the cage between 4 July and 31 October (Fig. 1). Mean values for each day were calculated and plotted. As water temperature profiles inside the cage and outside nearby the cage were almost identical, in the following we focus on the results obtained inside the cage.

Statistics

PKD prevalence and incidence were calculated as the sum of *T. bryosalmonae*-positive animals per group divided by the total number of animals per group (percentage). Parasite infection intensity was calculated by the sum of values for infection intensity per fish divided by the total number of infected animals. Mean pathology score was calculated by the sum of pathology scores per fish divided by the total number of affected animals.

Statistical differences in incidence and prevalence between groups (grayling versus brown trout, caged versus wild brown trout) were calculated using the chi-square test. Additionally, differences in infection intensity were tested between wild and caged animals using the chi-square test. To estimate mortality of non-infected caged fish, the number of PKD-free dead fish was divided by the total number of fish diagnosed to be PKD free (percentage). To estimate daily mortality rates, this number was divided by the

number of days of the experimental period. The mortality of PKD-infected brown trout over 5 d periods was then tested against the estimated daily mortality of the PKD-free animals using binomial tests. To test the correlation between size, condition index and PKD infection status, Spearman rank correlation tests were applied. In all tests, p-values < 0.05 were considered significant. All analyses were carried out in R, version 3.1 (www.r-project.org/) using the packages rcmdr, car and Rcmdmisc.

RESULTS

Water temperature

Water temperature exceeded 15°C over 41 non-consecutive days over the whole experimental period. At the beginning of August, temperature constantly exceeded 15°C for a maximum of 11 consecutive days (Fig. 1).

Cage experiment

At the start of the experiment, the length of grayling varied between 5.7 and 7.0 cm, and that of brown trout between 7.5 and 9.4 cm. None of the grayling and brown trout sampled at the beginning of the experiment before exposure to river water showed detectable *Tetracapsuloides bryosalmonae* DNA. Fish that were kept as references for the negative infection status in the farm and hatchery were also confirmed to be free of *T. bryosalmonae* DNA by real-time PCR at the end of the experiment. In none of these animals were any histological lesions identified. No mortality was recorded in the reference groups.

Grayling

Mortality

Inside the cage in the River Wutach, 41 grayling (40%) died during the course of the experiment (Fig. 2). While about half of these animals (22) died during a period of 2 wk at the end of July/beginning of August, i.e. within the first 3 wk after stocking, subsequent mortality of remaining grayling (19 animals) was equally distributed over the rest of the experimental period (Fig. 2a). None of the dead grayling showed either macroscopical signs of PKD or any histological kidney pathology typical for PKD or presence of *T. bryosalmonae* DNA. Instead, in 24 of the 41 grayling there were multiple bacterial colonies in kidney, spleen and perivisceral fat tissue (Fig. 3e). Multiple areas of necrosis were obvious in the vicinity of the bacterial colonies. Presence of *Aeromonas salmonicida salmonicida* was tested by PCR. However, no conclusive results were obtained (data not shown). Due to the absence of parasite DNA and PKD typical lesions, the mortality in graylings was not considered to be PKD related.

Infection status in surviving grayling

The surviving 62 grayling were euthanized after 88 d. Length varied between 6.0 and 15.5 cm, and weight between 2 and 8 g. Body condition was 0.56 (SD: 0.12), which falls within the normal range for grayling according to FishBase (Froese & Pauly 2016). None of the surviving grayling showed macroscopic signs of a PKD infection (Fig. 3a). Using histology, in one animal, small areas of interstitial proliferation and necrosis with single *T. bryosalmonae* were detected. The parasitic nature of the infection could be confirmed by IHC (Fig. 3g). Six additional grayling showed scattered small areas of necrosis in the renal interstitial tissue; however, no associated infectious agent could be determined. *Tetracapsuloides bryosalmonae* DNA was not detected by real-time PCR in any of the histologically or immunohistochemically negative grayling.

Brown trout

Mortality

Twenty-four brown trout died between the beginning of August and the beginning of October (Fig. 2). Seven brown trout that died during August showed neither macroscopic signs of PKD nor any histological kidney pathology nor presence of *T. bryosalmonae* DNA. In 5 of these fish, scattered bacterial colonies similar to the ones observed in grayling were obvious; however, infection intensity was lower than in grayling, i.e. only very few bacterial colonies were present. In animals that died at later time points, infection with *T. bryosalmonae* was evident in 15 out of 17 animals. These 15 animals showed mild to severe kidney lesions typical for an acute PKD infection (mean value pathology score: 2.9, SD: 0.8; mean value infection intensity: 2.6, SD: 0.9) and no

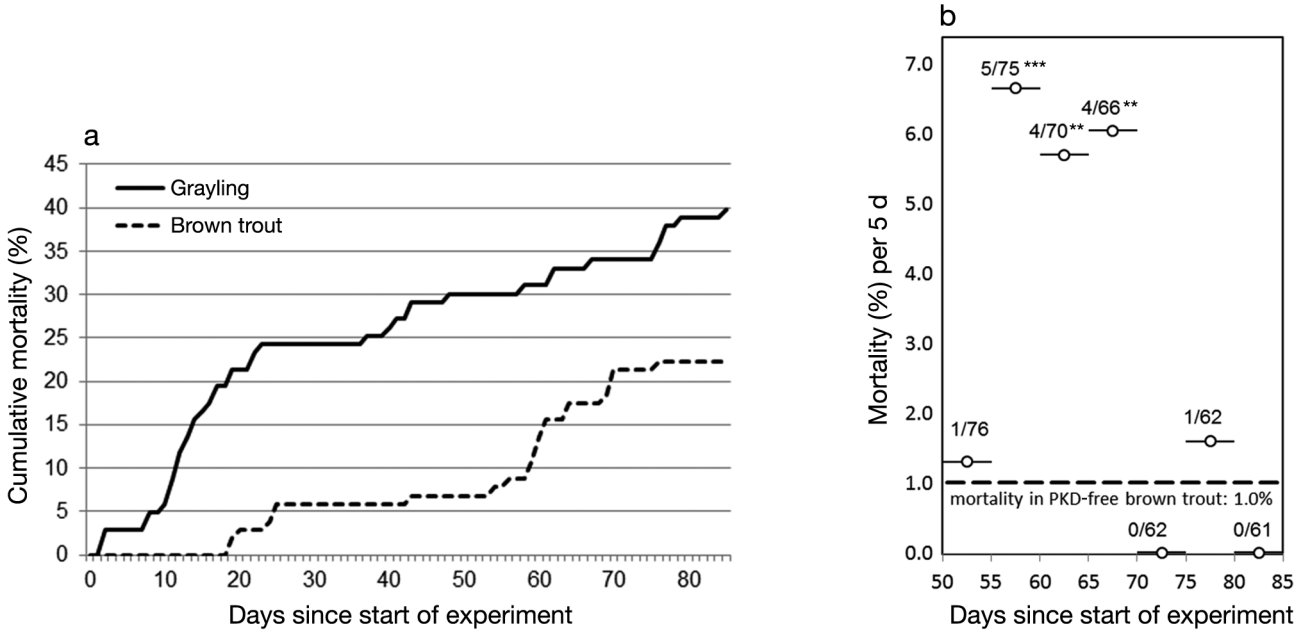

Fig. 2. (a) Grayling *Thymallus thymallus* and brown trout *Salmo trutta fario* cumulative mortality over the experimental period. Cumulative mortality in grayling shows a sharp increase in the first 3 wk, interpreted as stress-induced mortality, and a slower increase over the remaining period. Cumulative mortality in brown trout shows peak mortality between Days 50 and 66 post exposure, mainly associated with proliferative kidney disease (PKD) infections. (b) Mortality rates of brown trout in the cage experiment. Open symbols represent the mortality rate of PKD-positive animals over a 5 d period; values of 0 indicate that no animals died in a 5 d period because of PKD. Daily mortality of PKD-free animals was estimated over the period marked with the dashed line. The first 50 d were not considered, as animals in this period died without any signs of PKD. For information about the calculation of the mortality rates, see Tables S1 and S2 in the Supplement at www-int-res.com/articles/suppl/d123p193_supp.pdf. ***$p < 0.001$, **$p < 0.01$

other pathological lesions that could be associated with mortality. In 2 animals, neither histological, immunohistochemical or real-time PCR analyses revealed presence of parasites. In none of the animals were signs of a concurrent disease detected in these spontaneous deaths; therefore, mortality associated with PKD was calculated at 15% (15/103). These PKD-related mortalities occurred between 54 and 76 d post-exposure (dpe; Fig. 2).

When comparing mortality rates of *T. bryosalmonae*-infected and non-infected fish from the cage, the daily mortality rate was significantly higher in *T. bryosalmonae*-infected animals (55 to 70 d after the start of the experiment: binomial tests compared with the estimated daily mortality rate in PKD-free animals, $p < 0.004$; see Fig. 2). This mortality occurred shortly after the water temperature had remained above 15°C for several days. During the last 15 d of the experiment, only one PKD-positive fish died, which was not significantly different from the mortality of PKD-free animals in the same period (binomial test: $p = 0.84$).

When comparing mortality of grayling and brown trout, non-PKD-related mortality was significantly higher in grayling compared with brown trout (χ^2 = 6.50, df = 1, $p = 0.01$). PKD-related mortality was diagnosed in brown trout only (17 out of 103 = 15%) and not in graylings (0 out of 103) ($\chi^2 = 115$, df = 1, $p < 0.001$) (see below).

Infection status in surviving brown trout

Seventy-nine brown trout were euthanized after 88 dpe. Length varied between 7.0 and 15.0 cm, and weight between 3 and 46 g. Body condition was 1.14 (SD: 0.32), which falls within the normal range of brown trout according to FishBase (Froese & Pauly 2016). Seventy-three animals showed mild to severe macroscopical kidney changes (Fig. 3b). Sixty-one of the 79 surviving brown trout (77%) showed intact *T. bryosalmonae* by histology. In 3 additional animals, immunohistochemically positive parasites were detected in the tubular lumen and scattered in the renal interstitium. Therefore, the final percentage of PKD-positive surviving animals was 81% (64 out of 79 brown trout). There was no correlation between size, condition index and PKD infection status (Spearman rank correlation tests, $\rho = -0.15$, $p > 0.1$). Infection intensity in histologically and immunohisto-

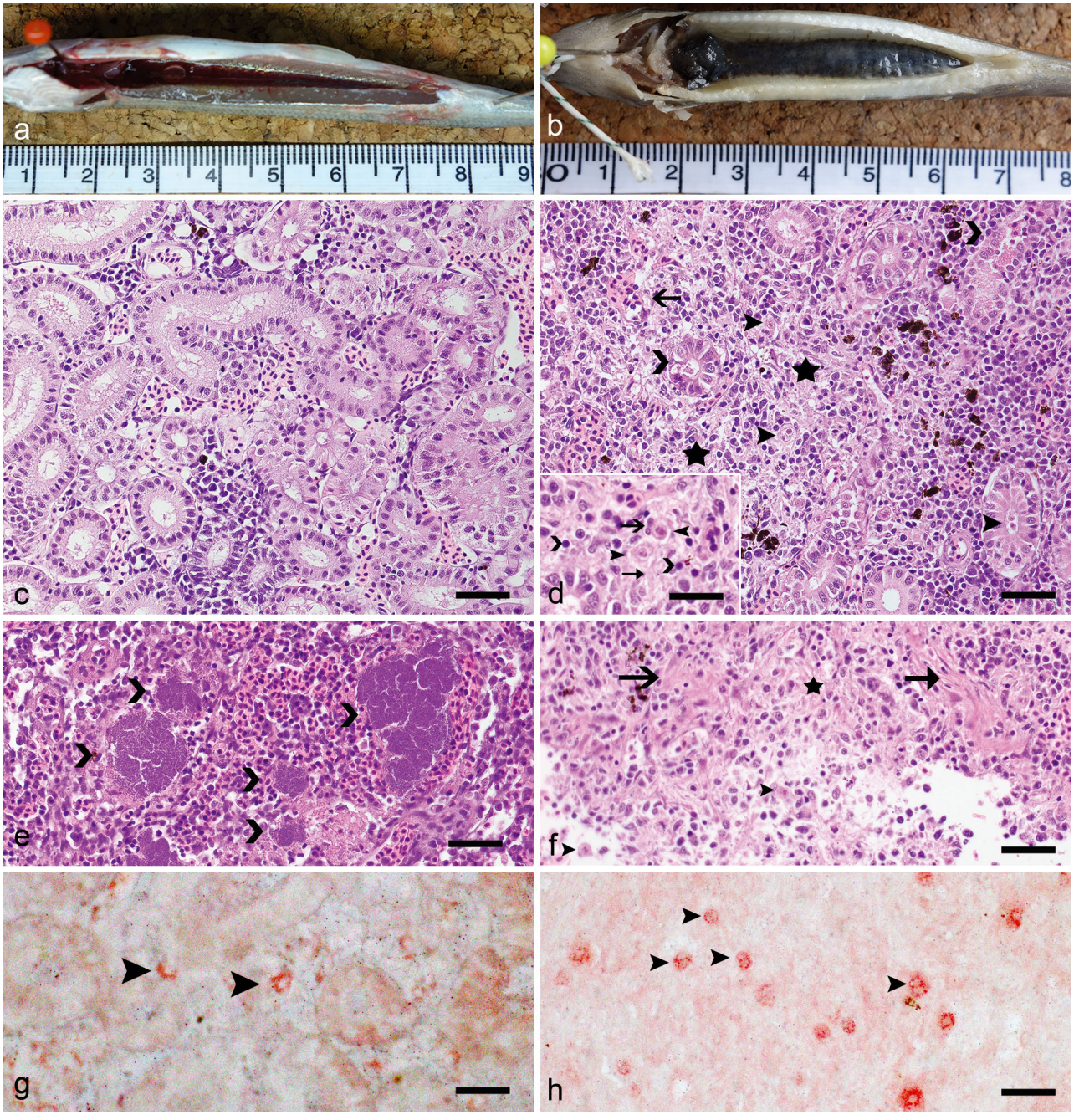

Fig. 3. (a,b) Macroscopic, (c,d,e,f) histological and (g,h) immunohistochemical appearance of surviving (a,c,e,g) grayling *Thymallus thymallus* and (b,d,f,h) brown trout *Salmo trutta fario* in the cage experiment. (a) Grayling, kidney with normal appearance. (b) Brown trout, kidney enlarged showing multiple greyish nodular lesions. (c) Grayling, no morphological alterations; HE stain. (d) Brown trout, acute inflammatory renal changes with interstitial necrosis characterized by cell debris and eosinophilic amorphous material (stars), vascular necrosis (arrow with open arrowhead) and multiple intralesional parasites (closed arrowheads), multiple tubuli were degenerated (tubulonephrosis) (open arrowhead); inset: higher magnification of 2 parasites (closed arrowheads), one parasite in surrounded by macrophages (arrow with open arrowhead), surrounding necrosis characterized by karyopyknosis (open arrowheads) and eosinophilic amorphous material (arrow with closed arrowhead); HE stain. (e) Grayling, spleen, multiple bacterial colonies (open arrowheads) surrounded by small amounts of eosinophilic material (necrosis) and haemorrhage; HE stain. (f) Brown trout, large vessel showing thrombosis (star), vascular wall (arrow with closed arrowhead), necrosis of vascular wall (arrow with open arrowhead), thrombus consisting of inflammatory cells, often degenerated, and parasites (closed arrowheads); HE stain. (g) Grayling, in one animal scattered parasites were visible with immunohistochemistry (closed arrowheads). (h) Brown trout, immunohistochemistry showing multiple parasites in interstitial tissue. Scale bars: (c,d,e,f) 25 µm; (inset, g,h) 10 µm

chemically positive surviving trout varied between scattered and moderate to severe (Fig. 3h) at a mean intensity of 0.9 (SD: 1.2). Using real-time PCR, no additional cases of *T. bryosalmonae* DNA positive animals were detected. Five randomly selected, histologically positive cases were all confirmed positive for *T. bryosalmonae* DNA by real-time PCR, using two 20 µm sections of the paraffin-embedded material. Comparing PKD incidence in the animals that died spontaneously and the surviving animals, there were no significant group differences ($\chi^2 = 1.61$, df = 1, p = 0.2). This could be confirmed when comparing animals that died after the initial phase of adaptation to cage conditions (after 14 September) with the surviving brown trout ($chi^2 = 0.88$, df = 1, p = 0.35). Also, infection intensity did not differ significantly between dead and surviving brown trout (intensity grouped in 3 levels: $\chi^2 = 1.21$, df = 2, p = 0.55)

Seventy-two of the surviving brown trout presented different patterns of kidney pathology. Forty-two trout showed acute renal changes with extrasporogonic parasites in the interstitium and intravascular thrombi composed of parasite stages, fibrin and mainly macrophages and few lymphocytes (Fig. 3d,f). Twenty-two animals showed chronic active changes such as interstitial fibrosis, tubulonephrosis and tubuloneogenesis together with an acute response to the disease as described above. In these animals, parasites were present in the interstitium and in the vessels, and also in the tubular lumen (Fig. 3d). However, detectable numbers of intratubular parasites were low. In 3 animals, kidney changes were already chronic with fibrosis, tubulonephrosis and tubuloneogenesis. In one animal, just an increased amount of tubuloneogenesis was visible, also classified as chronic lesion. In none of these 4 brown trout showing chronic changes were parasites detectable histologically or immunohistochemically, nor was there parasite DNA detectable by real-time PCR. No signs of concomitant disease were present in any of the 79 animals.

Overall, PKD incidence was 77% (79 out of 103 exposed brown trout) after 3 mo of exposure (Table 1), which was not significantly different to PKD prevalence in wild brown trout (see below).

Field sampling

Infection prevalence, intensity and renal pathology

In the Wutach, at the upstream and downstream locations, PKD prevalence was 60% and 91%, respectively (Table 1). Infection intensity varied between scattered (grade 1) and severe (grade 6), with mean values of 3.8 and 4.1, respectively (Table 1). At the upstream location, infection with *T. bryosalmonae* was mostly associated with moderate to severe acute kidney lesions (Table 1); in one animal, lesions were chronic, active and parasite numbers were low. The mean pathology score was 2.7 (SD: 2.5; Table 1). At the downstream location, 9 infected animals showed acute kidney lesions. The mean pathology score was 3.9 (SD: 1.8). One animal showed acute renal lesions and an interstitial severe fibrosis, interpreted as a chronic active lesion. At the upstream

Table 1. *Thymallus thymallus* and *Salmo trutta fario* field sampling and cage experiment, including location of sampling, prevalence, infection intensity (means ± SD) and associated renal pathology (means ± SD), total mortality and PKD-related mortality. nk: not known. Histopathological changes of the kidney were graded as 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe). Presence of *Tetracapsuloides bryosalmonae* was classified as 0 (no parasites present on whole slide), 1 (single parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a magnification of 200 to 400×

Location		Species	n	Prevalence/ incidence (%)	Infection intensity	Renal pathology	Mortality (%)	PKD in dead animals (%)
Field sampling								
Wutach	Upstream	Brown trout	10	60	3.83 ± 1.47	4.5 ± 1.22	nk	nk
	Downstream	Brown trout	11	91	3.88 ± 1.55	4.3 ± 1.34	nk	nk
Cage experiment								
Fish farm	Starting reference	Grayling	10	0	0 ± 0	0 ± 0	0	0
		Brown trout	10	0	0 ± 0	0 ± 0	0	0
Fish farm	Negative reference	Grayling	10	0	0 ± 0	0 ± 0	0	0
		Brown trout	10	0	0 ± 0	0 ± 0	0	0
Wutach	Caged animals	Grayling	103	1	0 ± 0	2.67 ± 2.04	40	0
		Brown trout	103	77	2.06 ± 1.05	2.92 ± 1.13	23	15

and downstream locations, parasites were located in the interstitial tissue and in the vessels in acute cases; in the chronic active cases, parasites were additionally found in the tubular lumen.

Chi-square analysis comparing PKD prevalence between field samples and the PKD incidence in the cage showed no significant group differences (χ^2 = 0.038, df = 1, p = 0.84). However, when comparing infection intensity (pooled into 3 categories: category 1 [mild infection rate] = intensity 1 and 2; category 2 [moderate infection rate] = intensity 3 and 4; category 3 [severe infection rate] = intensity 5 and 6), significant differences were obvious between field samples and surviving brown trout, with brown trout caught in the wild showing a significantly higher infection intensity compared with caged animals (χ^2 = 31.4, df = 2, p < 0.00001). The same was true for renal pathology (Table 1).

DISCUSSION

Species-specific differences seem to be present in respect to sensitivity to infections with *Tetracapsuloides bryosalmonae*. Grayling were recorded as hosts for *T. bryosalmonae* (Feist & Bucke 1993, Hedrick et al. 1993, Grabner & El Matbouli 2008), with pathology similar to that described for rainbow and brown trout (Grabner & El Matbouli 2008). However, to the best of our knowledge there have been no previous studies investigating incidence and mortality due to PKD in grayling. Therefore, the present study aimed to investigate incidence and mortality in grayling in comparison to identically exposed brown trout and to compare pathological lesions induced by infection. Experiments were performed under field conditions to examine the possibility of increased mortality in these species under the conditions present in the specific river stretch. Therefore, other stress factors possibly influencing the pathogenesis of *T. bryosalmonae* cannot be ruled out.

Whereas incidence in caged brown trout was high (77%), in identically exposed grayling, only one animal was found to be PKD positive (incidence: 1%). Even by means of real-time PCR, no additional *T. bryosamonae* DNA positive grayling kidney could be detected. However, it cannot be excluded that more grayling became infected during the course of the experiment without showing clinical signs or mortality and clearing the infection before being sampled. Furthermore, the rather late start of the experiment might have influenced the infection rate. However, as brown trout and grayling were exposed in parallel, brown trout experienced the same conditions; infections between the 2 species in the cage are comparable.

Mortality over the whole experimental period was significantly increased in grayling in comparison to brown trout. However, grayling mortality was not considered to be PKD related mainly for 2 reasons: (1) no evidence of a *T. bryosalmonae* infection nor any PKD-related alterations was found in dead grayling and (2) according to several laboratory infection trials, PKD-related mortality does not start within the first 3 wk of exposure, even in heavily infected fish (e.g. Bettge et al. 2009). As this mortality in grayling started shortly after stocking, this might be related to stress due to the change of conditions from the farm to the river. Further, multiple bacterial colonies and associated necrotic areas were present. Real-time PCR revealed these bacterial colonies to be negative for *Aeromonas salmonicida salmonicida* (data not shown). Other bacteria seem to be the responsible agents, or these bacteria are post-mortem overgrowth. If occurring intra vitam, this bacterial infection might have further added to the increased mortality rate in the first weeks of the experiment. In contrast, mortality in brown trout was significantly increased after the warm water period, i.e. during a period in which PKD infection in this species could be demonstrated. As no evidence for other infectious agents could be found, death due to the *T. bryosalmonae* infection is likely.

Previous studies hypothesized that increased PKD-related mortality is a major contributor to the decline of brown trout populations (Wahli et al. 2008, Zimmerli et al. 2007, Schmidt-Posthaus et al. 2013), and YOY brown trout seemed to be especially affected (Burkhardt-Holm et al. 2005, Hari et al. 2006). In an earlier study exposing brown trout YOY to river water of the Wutach, mortality stayed as low as 15% (Schmidt-Posthaus et al. 2015), despite the fact that 69% of caged fish became infected during the experimental period. Results of the present study further strengthen these data, with an incidence of 77% and PKD-associated mortality of only 15%. This mortality started 11 d after the temperature exceeded 15°C (daily mean value) for the last time. The significant differences in infection intensity and renal pathology between sampled wild trout and caged animals observed in this study might be explained by the time gap of 28 d between sampling in the field and termination of the cage experiment. However, it cannot be excluded that in the field under continuous exposition to the parasite load and under changing temperature conditions in the course of the river, mortality

could be higher compared with in our experiment. Further, the cage experiment was started in July. It is likely that wild fish at this time point had already been exposed for several weeks to infective *T. bryosalmonae*, which might have resulted in more intense kidney alterations.

In the River Wutach, the grayling population has declined dramatically in the last 35 yr and PKD infections have been discussed as a possible contributing factor, similar to events in brown trout populations (Burkhardt-Holm et al. 2005). However, the results of this study could not confirm this hypothesis, as under the tested conditions, incidence in grayling was only 1% and grayling mortality in the cage was not associated with PKD infection. As this was a single experiment, it cannot be ruled out that the situation would present differently in another year with other temperature regimes. However, our results suggest that in the River Wutach, PKD is not a major contributing factor to the severe decline of the native grayling population.

Our results give clear evidence that species-specific differences exist in susceptibility to infections with *T. bryosalmonae*. Under the tested conditions, PKD incidence in grayling was only 1%, whereas the disease incidence in parallel-exposed brown trout was as high as 77%. Furthermore, *T. bryosalmonae* infection-related pathology in grayling was minimal whereas brown trout showed moderate renal lesions, with granulomatous nephritis, necrotizing vasculitis and thrombosis. Grabner & El Matbouli (2008) described similar lesions in grayling and rainbow trout with severe renal swelling, hematopoietic proliferation and numerous interstitial parasites. In their experiment, fish were co-habitated with infected bryozoan colonies for 2 wk at 15°C (Grabner & El-Matbouli 2008). Therefore, conditions cannot be compared directly. Exposure time was shorter compared with our experiment, but in contrast to our study, temperature remained stable at 15°C over a prolonged period (Grabner & El Matbouli 2008). Additional research is needed to confirm these apparently conflicting findings with the data from the literature, including other river systems and with grayling strains of origin other than that from the Rhine investigated in this study. Further, different environmental conditions might also influence susceptibilities of species. Additionally, other fish species or strains should be included in species comparisons.

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