

Severe, chronic proliferative kidney disease (PKD) induced in rainbow trout *Oncorhynchus mykiss* held at a constant 18°C

D. J. Morris*, H. W. Ferguson, A. Adams

Scottish Fish Immunology Research Centre, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

ABSTRACT: Proliferative kidney disease (PKD), caused by the myxozoan parasite *Tetracapsuloides bryosalmonae*, is well documented as a seasonal disease of rainbow trout *Oncorhynchus mykiss*. Water temperatures influence the course of the infection both within the fish and the invertebrate host, the recovery of fish from the disease being accelerated with decreasing water temperatures. During this study, groups of rainbow trout were held at a constant temperature (18°C) for a sustained period of time following initial exposure to *T. bryosalmonae*. While the majority of these fish had recovered from the clinical disease after 9 mo, 10% remained infected, showing clinical signs of disease. A histological study revealed that the majority exhibited very high parasite loads and unusually severe symptoms of PKD. This demonstrates that while most rainbow trout can recover from PKD independent of water temperature, there exists a sub-population that cannot.

KEY WORDS: *Tetracapsuloides* · Myxozoa · Malacosporea · Temperature

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INTRODUCTION

Proliferative kidney disease (PKD) is a seasonal disorder of cultured salmonids in Western Europe and North America. It is caused by the extrasporogonic stage of the myxozoan parasite *Tetracapsuloides bryosalmonae*, and is characterised by nephromegaly, splenomegaly, hypergammaglobulinemia and anaemia (Ferguson & Needham 1978, Foott & Hedrick 1990). Fish that recover from clinical PKD are immune to further outbreaks of the disease (Foott & Hedrick 1987).

The severity of PKD and the subsequent recovery of fish from the disease are influenced by water temperature. Water temperatures over 12°C are known to induce the clinical disease. The disease progresses more rapidly and becomes more severe as temperatures increase (Clifton-Hadley et al. 1986). Conversely, as the water temperature decreases so the speed of recovery increases (Ferguson 1981). It is thought that temperature impacts the multiplication of the parasite

within the host (Gay et al. 2001). Low-temperature infection regimes, whereby rainbow trout have accumulated 1300 degree-days at an average water temperature of 10 to 11°C, have demonstrated that the fish can acquire a resistance to the parasite while avoiding the clinical disease (de Kinkelin & Lorient 2001).

Several studies have confirmed that rainbow trout will recover from PKD when maintained at relatively high ($\geq 15^{\circ}\text{C}$) water temperatures (Ferguson 1981, Kent & Hedrick 1986, Clifton-Hadley et al. 1987, MacConnell et al. 1989, Chilmonczyk et al. 2002). However, only the studies of Ferguson (1981) and Kent & Hedrick (1986) have reported the recovery of all fish from the clinical disease at the end of the trials. In our laboratory, we have noticed that within groups of fish that have recovered from PKD, a few individuals still manifest the clinical symptoms. Here we report on an experiment to determine whether all rainbow trout can recover from PKD when held at permissive temperatures over an extended period of time.

*Email: d.j.morris@stir.ac.uk

MATERIALS AND METHODS

Experimental facilities. The rainbow trout were maintained within the Aquatic Research Facility (ARF) at the University of Stirling. This facility is specific pathogen-free, and uses temperature-controlled, de-chlorinated, mains tap water to feed experimental 150 l flow-through tanks. All fish introduced into the experimental aquarium facilities were prophylactically treated with a 1 h flush of 200 ppm formaldehyde and an intraperitoneal injection of 10 mg kg⁻¹ body wt oxytetracycline to remove ectoparasitic and possible concomitant bacterial infections.

Fish infection and maintenance. We used 2 groups of 20 and 24 fingerling rainbow trout, respectively. The first group was obtained in September from a PKD enzootic fish farm. These fish (mean weight 20 g) were from a batch introduced into the farm the previous month from the hatchery. Infection with *Tetracapsuloides bryosalmonae* within the batch was confirmed by kidney imprints stained with Rapi-Diff (London Laboratories), in conjunction with an assessment of the level of kidney-swelling as described by Clifton-Hadley et al. (1987). The second group of rainbow trout (mean weight 10 g) was obtained from a hatchery with no history of PKD. These fish were experimentally infected with *T. bryosalmonae* by an intraperitoneal injection of *T. bryosalmonae*-infected kidney homogenate as described by Kent & Hedrick (1985). After 7 wk, 4 fish from this group were killed and examined to confirm successful transmission of *T. bryosalmonae*.

All fish were introduced into experimental tanks in the ARF, fed with proprietary feed, and held for 40 wk at a constant 18°C, in an 18:6 h day:night regime. Naïve sentinel rainbow trout were also maintained in the ARF on the same water supply during the course of the experiment. Samples from these fish were routinely examined for evidence of *Tetracapsuloides bryosalmonae* infection.

Sampling. At the end of the study all of the fish were anaesthetised using benzocaine, followed by severing of the spinal cord just behind the head. Samples of kidney, spleen, gill, intestine, liver and skeletal muscle at the level of the lateral line were fixed in 10% buffered formalin. These were routinely processed into paraffin wax for histology, and sectioned and stained using haematoxylin and eosin Y and Gomori's trichrome. Selected sections were immunostained either with the *Tetracapsuloides bryosalmonae*-specific antibody P01 (Aquatic Diagnostics) as specified by the manufacturer, or by using monoclonal antibody MAb B4. This latter antibody reacts specifically with the sporogonic stages of the parasite (Morris et al. 1997).

RESULTS

Examination of fish (n = 20) sampled at the time the naturally exposed rainbow trout were collected, indicated a high infection level with *Tetracapsuloides bryosalmonae* at the farm site, as all kidneys possessed extrasporogonic parasites and the majority (n = 14) displayed clinical symptoms of kidney swelling (Grade 1). This concurred with previous studies at this farm site that demonstrated a 100% prevalence of the disease within stocks introduced during the summer months. The 4 fish sampled from the experimentally infected group after 7 wk were all positive for *T. bryosalmonae*. During the course of the study no fish died or displayed any overt symptoms of distress.

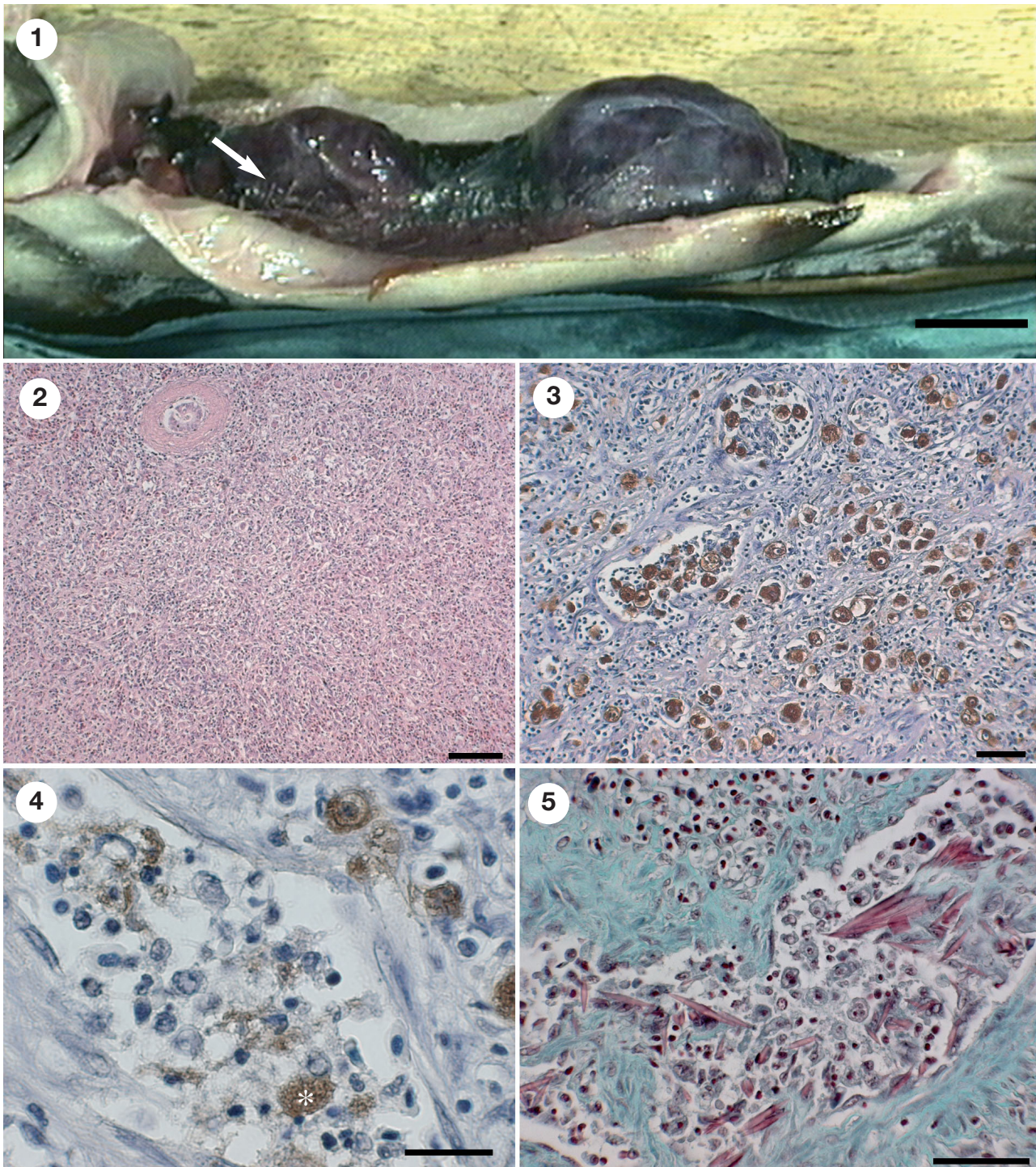
When the 2 groups of fish were sampled at the end of the study, a total of 4 fish (2 from each group) were found to have gross, internal pathological changes attributed to chronic PKD. All other fish appeared disease-free. Of the diseased fish, 1 had a kidney swelling of Grade 2 with pathological changes consistent with this stage of PKD, while the other 3 displayed manifestations of unusually severe disease. Of these, 1 from each group had an extremely enlarged posterior kidney, while the third, from the injected group, had marked splenomegaly.

Gross lesions

Externally, the 3 fish with marked manifestations of the disease all had a roughened, nodular dermis along the flank and pale, watery blood. While the abdominal swelling typically associated with PKD was not noted, the body wall of the 2 fish with nephromegaly nevertheless was markedly distended at the level of the posterior kidney.

Internally the most notable change in 2 of the fish sampled was the massive enlargement of the posterior kidney, measuring ~3.5 cm in diameter (Fig. 1). Marked swelling was also present in the anterior kidney and to a lesser extent also in the middle kidney. The kidney was mostly dark red, although the posterior portion had grey mottling and a thickened capsule. Apparent degeneration of the peritoneum was noted, as the ribs remained in position when the flank was retracted during dissection. The spleens of these fish were enlarged, with a mottled surface, resembling those of fish with a Grade 4 PKD.

In the fish with the marked splenomegaly, the spleen was roughly cylindrical, 8 cm long and 1 cm in diameter, extending nearly the entire length of the body cavity. It had a grey appearance with red mottling. The kidney of this fish was swollen in accordance with a Grade 3 PKD.



Figs. 1 to 5. *Oncorhynchus mykiss* infected with *Tetracapsuloides bryosalmonae*. Fig. 1. Ventral view of dissected rainbow trout with major nephromegaly. Arrow indicates position of ribs after dissection (scale bar = 2.0 cm). Fig. 2. Kidney architecture replaced by granulomatous reaction; near top of photo is a renal tubule surrounded by fibrous tissue; haematoxylin and eosin (scale bar = 100 μ m). Fig. 3. Immunohistochemical staining revealing large numbers of *T. bryosalmonae* extrasporogonic stages (brown staining) in granuloma (scale bar = 50 μ m). Fig. 4. Immunohistochemical localisation of released parasite antigen (brown staining) within vessel of granuloma; *: intact parasite (scale bar = 20 μ m). Fig. 5. Fusiform crystals, parasites and immune cells within vessel composed of large quantities of collagen; Gomori's trichrome (scale bar = 50 μ m)

The cut surface of both spleen and kidney appeared to be primarily composed of a yellow/grey centre surrounded by diffuse red tissue. Ascitic fluid was present in the body cavity and all of the fish appeared anaemic, with pale gills and liver. The liver had areas of ecchymotic haemorrhage under the capsule.

Histopathology

The 2 fish with severe nephromegaly had similar lesions. The normal architecture of the posterior kidney was obliterated with an ongoing granulomatous nephritis and progressive fibrosis (Fig. 2). A few isolated tubules or intact glomeruli were still present within the reaction, usually surrounded by layers of fibrous tissue. The granulomata was of markedly vascular nature, and the vessels contained inflammatory cells, extrasporogonic stages of *Tetracapsuloides bryosalmonae* and a few erythrocytes. The stroma of the granulomata consisted of fibroblasts, epithelioid cells, erythrocytes and small mononuclear cells. Immunostaining revealed that very high numbers of extrasporogonic parasites were also present (Fig. 3). Localisation of the P01 antibody also revealed areas where there appeared to be breakdown of the extrasporogonic parasite with a corresponding release of parasite constituents into the stroma and granuloma vasculature (Fig. 4). Immunostaining with MAb B4 demonstrated the presence of sporogonic stages within a few of the remaining kidney tubules.

Localised areas, consisting of large quantities of collagen, formed vessels that, in addition to parasites and inflammatory cells, also contained numerous fusiform crystals, presumably of haemoglobin (Fig. 5). Renal portal blood vessels contained large amounts of cellular and proteinaceous debris along with small mononuclear cells, extrasporogonic parasites and erythrocytes. Towards the periphery of the granulomata the tissue differentiated into more organised renal tissue, displaying haematopoietic hyperplasia together with a few extrasporogonic parasites and nephros, but no distinct boundary could be observed between this and the granulomatous tissue (Fig. 6). Extrasporogonic parasites were noted infiltrating into the neighbouring haematopoietic tissue with an associated nephritis. Parasite infiltration into the capsule of the kidney was also noted. Occasional extrasporogonic parasites, erythrocytes and small mononuclear cells were present in the lumen of the urinary bladder. The anterior kidney had pathological changes similar to those in the posterior kidney, with advanced granulomata formation.

In contrast, tissue from the middle kidney revealed only a moderate interstitial hyperplasia that displaced

the excretory elements. Relatively few extrasporogonic and sporogonic parasites were present within this tissue, and the overall appearance resembled that described for Grade 1 PKD (Clifton-Hadley et al. 1987). A similar fibro-granulomatous response was noted in the enlarged spleens.

For the solitary fish with dramatic splenomegaly, the splenic architecture was replaced with granulomatous inflammation, extrasporogonic parasites and fibrosis as noted for the severe nephromegaly above. Large pools of erythrocytes were also present, while the capsule was thickened. The kidney of this fish possessed haematopoietic hyperplasia and a moderate infiltration of parasites consistent with a Grade 2 PKD.

The fish with moderate Grade 2 renal swelling had changes consistent with this stage of the disease as described by Clifton-Hadley et al. (1987). In addition, 2 of the apparently healthy fish had occasional extrasporogonic parasites within the interstitium of their kidney associated with limited hyperplasia.

The livers of the 3 fish with severe symptoms all had similar lesions consisting of an ongoing infiltration of immune cells, parasites and fibrosis throughout the tissue resulting in a displacement and disorganisation of the hepatocytes (Fig. 7). Thrombi, composed of parasites and immune cells were present within many of the hepatic blood vessels, while immune cells and fusiform crystals were present in the bile ducts.

While a large number of gill lamellae contained solitary extrasporogonic parasites (~30%), epithelial hyperplasia or other immune reactions were rarely observed in this tissue.

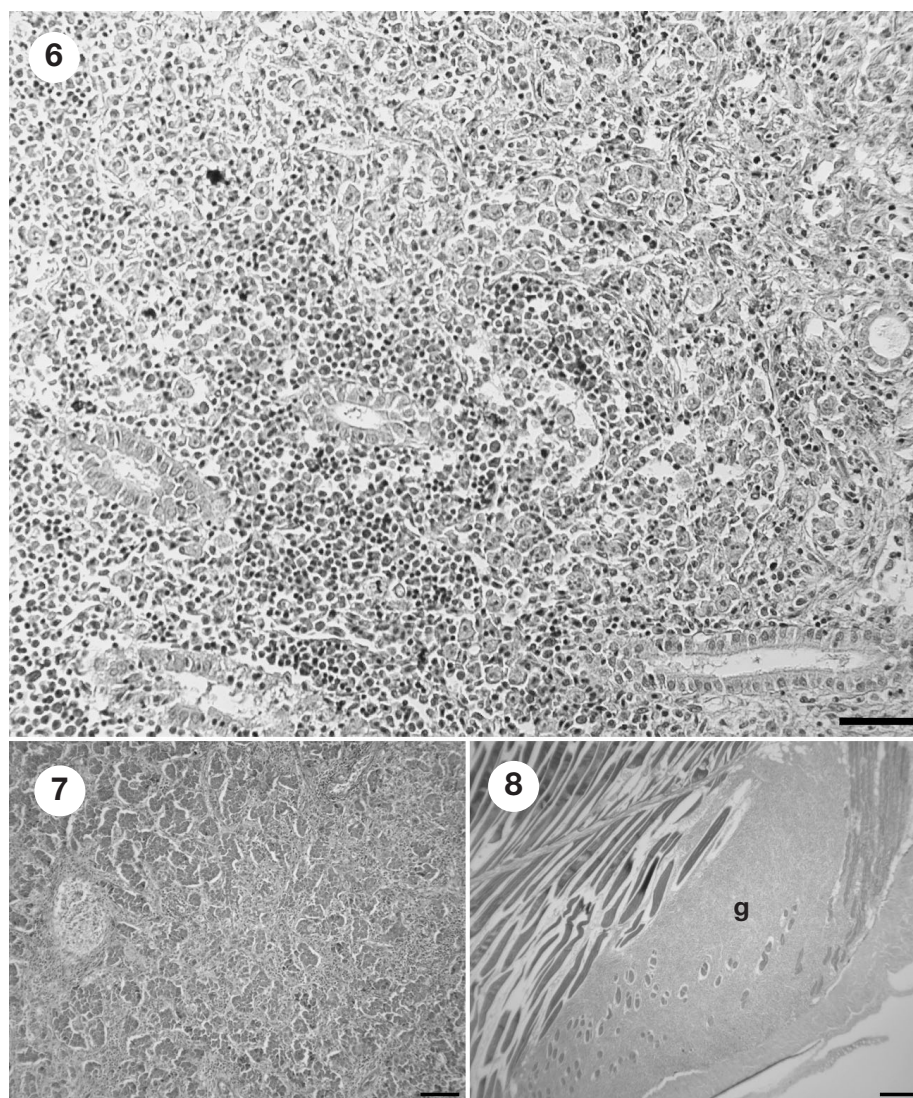
Numerous distinct granulomata, containing fibroblasts, immune cells and parasites were present both throughout the white muscle and immediately below the skin surface (Fig. 8). The granulomata often surrounded individual muscle fibres. On the periphery of the granulomata, fibre necrosis occasionally occurred. Infiltration of immune cells and parasites was also noted extending from the granulomata between the neighbouring fibres.

The intestine of the fish appeared normal, with only 1 extrasporogonic parasite being observed within a blood vessel of any of the fish examined.

DISCUSSION

The majority of rainbow trout in this study appeared to recover entirely from PKD, corroborating previous studies on the course of the infection (Kent & Hedrick 1986). However, a minority did not recover, displaying either clinical or conversely sub-clinical PKD. The observation that in some fish the onset of PKD may be delayed, and they may have differing levels of immune

Figs. 6 to 8. *Oncorhynchus mykiss* infected with *Tetracapsuloides bryosalmonae*. Fig. 6. Infiltration of extrasporogonic parasites (top) into neighbouring haematopoietic tissue (bottom); haematoxylin and eosin (scale bar = 100 μ m). Fig. 7. Disorganisation of liver hepatocytes with diffuse inflammation and fibrosis; haematoxylin and eosin (scale bar = 100 μ m). Fig. 8. Granuloma (g) directly beneath skin; isolated myofibrils are present within granuloma; Gomori's trichrome (scale bar = 250 μ m)



response during the infection, has been previously documented (Chilmonczyk et al. 2002). However, the reasons for this differential development and response are unclear. Although host genetic factors may be involved, genetically homogenous populations have been documented as still displaying such disparities during the development of the disease (de Kinkelin & Lorient 2001). Since similar effects have also been noted during infection studies using parasite transmission via intraperitoneal injection, initial parasite burden is also unlikely to be a factor. Therefore, in addition to host genetic factors, the most plausible explanation is unquantifiable tank effects such as hierarchy acting upon the fish at an individual level. These effects may also contribute to the chronic disease observed during this study.

In previous studies, rainbow trout displaying increasingly severe PKD at the end of a typical episode

have not been reported. While tank effects may explain the differential ability of the fish to recover from clinical PKD, they do not in themselves explain the unusual severity of the disease observed during this study. We consider that there are 2 possible reasons for the chronic severe disease: the prior removal of pathogens from the fish and the maintenance of a permissive water temperature.

Mortalities associated with PKD are usually attributed to additional environmental stressors or disease (Smith et al. 1984). Affected fish are thought to be particularly sensitive to bacterial challenge due to the depression of granulocyte activity (Chilmonczyk et al. 2002). Although some studies have utilised pathogen free water to maintain the fish, no long-term study on the course of the disease has been reported as prophylactically treating for concomitant infections. While, in the present study, these treatments may have dis-

rupted the initial course of the infection, they would not have persisted for a prolonged period, and therefore it is unlikely that the chronic severe disease observed was a direct result of the treatment. Rather, due to the extreme manifestations of disease encountered, it is reasonable to assume that any additional infection in these fish would have killed them. Therefore, in a situation in which pathogens were not strictly controlled, these fish would undoubtedly have died. This would probably have occurred before the severity of the disease had reached the extreme stage noted here, and therefore no unusual pathology would have been noted.

The artificial maintenance of a permissive temperature may also be a factor that resulted in the chronic disease. It is accepted that the maintenance of a high temperature during *Tetracapsuloides bryosalmonae* infection will result in the disease progressing more rapidly while delaying the ability of rainbow trout to recover (Ferguson 1981, Clifton-Hadley et al. 1986). De Kinkelin & Lorient (2001) suggested that maintaining permissive temperatures (10 to 11°C) over a period of time allowed these fish to develop resistance while avoiding the clinical symptoms of the disease. However, during their study 2 fish that were sampled 236 d after initial infection displayed major nephromegaly and splenomegaly. Due to the severity of the inflammatory response shown by these fish it is possible that they had symptoms similar to the severe chronic disease described in the present study. This would suggest that sub-populations of rainbow trout will develop chronic, severe PKD if held at any permissive temperature. This has implications for studies examining the response of rainbow trout to successive exposures to *T. bryosalmonae*, in which it will be advisable to reduce the temperature to below those known to be permissive for the parasite (<9°C), thus ensuring the fish are free from extrasporogonic parasite infection during the subsequent exposure.

Hepatitis and myositis were both seen in *Oncorhynchus mykiss* severely infected fish in this study. Farmed rainbow trout in Spain also showed granulomatous myositis caused by *Tetracapsuloides bryosalmonae* towards the end of a PKD outbreak in October (Feranadez-de Luco et al. 1997, Peribáñez et al. 1997). Although the percentage of individuals involved was relatively high, such unusual lesions may relate to the fish having clinical PKD over an extended period by being held at permissive temperatures in conjunction with improved water conditions. As the welfare of farmed rainbow trout increases, allowing diseased fish to survive longer into the autumn, it is to be expected that more cases of unusual and severe manifestations of PKD will be reported.

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LITERATURE CITED

- Chilmonczyk S, Monge D, de Kinkelin P (2002) Proliferative kidney disease: cellular aspects of the rainbow trout, *Oncorhynchus mykiss* (Walbaum), immune response to parasitic infection. *J Fish Dis* 25:217–226
- Clifton-Hadley RS, Richards RH, Bucke D (1986) Proliferative kidney disease (PKD) in rainbow trout *Salmo gairdneri*: further observations on the effects of water temperature. *Aquaculture* 55:165–171
- Clifton-Hadley RS, Bucke D, Richards (1987) A study of the sequential clinical and pathological changes during proliferative kidney disease in rainbow trout, *Salmo gairdneri* Richardson. *J Fish Dis* 10:335–352
- de Kinkelin P, Lorient B (2001) A water temperature regime which prevents the occurrence of proliferative kidney disease (PKD) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 24:489–493
- Ferguson HW (1981) The effects of water temperature on the development of proliferative kidney disease in rainbow trout, *Salmo gairdneri* Richardson. *J Fish Dis* 4:175–177
- Ferguson HW, Needham EA (1978) Proliferative kidney disease in rainbow trout *Salmo gairdneri* Richardson. *J Fish Dis* 1:91–108
- Fernández-de-Luco D, Peribáñez MA, García L, Castillo JA (1997) Granulomatous myositis in rainbow trout *Oncorhynchus mykiss* affected by proliferative kidney disease (PKD). *Dis Aquat Org* 31:49–54
- Foott JS, Hedrick RP (1987) Seasonal occurrence of the infectious stage of proliferative kidney disease (PKD) and resistance of rainbow trout, *Salmo gairdneri* Richardson, to reinfection. *J Fish Biol* 30:477–483
- Foott JS, Hedrick RP (1990) Blood parameters and immune status of rainbow trout with proliferative kidney disease. *J Aquat Anim Health* 2:141–148
- Gay M, Okamura B, de Kinkelin P (2001) Evidence that infectious stages of *Tetracapsula bryosalmonae* for rainbow trout *Oncorhynchus mykiss* are present throughout the year. *Dis Aquat Org* 46:31–40
- Kent ML, Hedrick RP (1985) Transmission of the causative agent of proliferative kidney disease (PKD) with the blood and spleen of infected fish; further evidence that the PKX parasite belongs to the phylum Myxozoa. *Bull Eur Assoc Fish Pathol* 5:39–42
- Kent ML, Hedrick RP (1986) Development of the PKX myxosporean in rainbow trout *Salmo gairdneri*. *Dis Aquat Org* 1:169–182
- MacConnell E, Smith CE, Hedrick RP, Speer CA (1989) Cellular inflammatory response of rainbow trout to the protozoan parasite that causes proliferative kidney disease. *J Aquat Anim Health* 1:108–118
- Morris DJ, Adams A, Richards RH (1997) Studies of the PKX parasite in rainbow trout via immunohistochemistry and immunogold electron microscopy. *J Aquat Anim Health* 9:265–273
- Peribáñez MA, Luco DF, García L, Castillo JA (1997) The prevalence of proliferative kidney disease from the kidney and muscle of rainbow and brown trout in Aragón (Spain). *Prev Vet Med* 32:287–297
- Smith CE, Morrison JK, Ramsey HW, Ferguson HW (1984) Proliferative kidney disease: first reported outbreak in North America. *J Fish Dis* 7:207–216