Examination of brown trout decline in the pre-alpine Isar river – Mortalities are linked with Proliferative Kidney Disease (PKD) and Proliferative Darkening Syndrome (PDS)

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ABBREVIATIONS

Δ cq-values - quantitation cycle of qPCR

°C – degree Celsius

Bp – base pairs

COX-2 – cyclooxygenase 2

DNA – deoxyribonucleic acid

e.g. – for example

EIBS – Erythrocytic Inclusion Body Syndrome

F. sultana – Fredericella sultana

G2 – gap phase of cell cycle

HSMI – Heart and Skeletal Muscle Inflammatory Disease

IL-8 – Interleukin 8

i.p. – intraperitoneal injection

IPN – Infectious Haematopoietic Necrosis

ITS-1 – internal transcribed spacer 1

KFU – kidney functional unit

M. *cerebralis – Myxobolus cerebralis*

M - phase – mitotic phase of cell cycle

MMC – melanomacrophage center

O. mykiss – Oncorhynchus mykiss

PDS – Proliferative Darkening Syndrome

PKD – Proliferative Kidney Disease

PKX – proper name for development stages of Tetracapsuloides bryosalmonae

PRV3 – piscine orthoreovirus type 3

PRV – Piscine Reovirus

qPCR – real time PCR

sdy gene - sexually dimorphic on the Y-chromosome

S-phase – synthase phase of cell cycle

S. trutta fario – Salmo trutta fario

TNF-α2 – tumor necrosis factor alpha 2

T. bryosalmonae – Tetracapsuloides bryosalmonae

USA – United States of America

UV – ultraviolet

VHS – Viral Haemorrhagic Septicaemia

I. INTRODUCTION

With the advent of climate change, global ecology and biodiversity are expected to underlie enormous alterations. Some of these changes may be more apparent than others, such as the decline of bees, which is discussed and recognized worldwide. Wildlife conservation programs are getting more popular, as in Bavaria where a referendum for bee protection obtained much attention and finally was successful. This referendum reflects the spirit of time we are living in – human beings are in fear of consequences due to global warming and start to consider counter measures.

However, wildlife protection does not stop at the water surface. A highly adapted and precious variability of species underwater do also need our attention and protection. Aquatic organisms, like fish or macrozoobenthos, are particularly affected by humanity's lifestyle. These highly sensitive species are distressed by disposals of human drugs, by reorganizations of rivers resulting from urban environmental shaping and by agricultural ejections in wastewater. Beside these environmental factors, new fish diseases are caused by emerging pathogens. The brown trout (*Salmo trutta fario*), the most famous native salmonid species in German river systems, suffers from declining natural populations. The Proliferative Darkening Syndrome (PDS) is observed as an emerging disease in various pre-alpine rivers in Bavaria during the last decades. Mass mortalities due to PDS diminish these natural brown trout populations in late summer months. In some rivers, populations decreased in such a high extent, that restocking with brown trout from hatcheries seemed the only option to sustain the species. Yet, the immunological system of hatchery fish may not be sufficiently adapted to the natural river environment with numerous potential pathogens. Bacterial infections with *Aeromonas salmonicida* are common. With increasing water temperatures in river systems, especially highly adapted species such as brown trout (*Salmo trutta fario*), are at higher risk. Brown trout prefer oxygen-rich and cold water. Increasing water temperature promotes environmental stress and may enhance infections with temperature-dependent pathogens like the myxozoan parasite *Tetracapsuloides bryosalmonae*, the causative agent of the

Proliferative Kidney Disease (PKD). Thus, fish pathogens are becoming increasingly important in research and better knowledge is urgently needed to better understand the infection pathways and to develop approaches of disease prevention in sensitive aquatic ecosystems.

In this study, severely diseased brown trout were collected within the pre-alpine Isar river in the center of Munich during the summer months in 2017 and 2018. Aim was to examine possible causes underlying the decline of this local brown trout population. PDS was suspected to trigger mass mortalities as reported by the Bavarian Fisheries Association (Landesfischereiverband Bayern e.V.) and local recreational anglers of the “Die Isarfischer München e.V.”. In addition, infections with *T. bryosalmonae* and resulting PKD were discussed as potential disease burden.

II. LITERATURE REVIEW

1. Brown Trout

The brown trout, *Salmo trutta fario*, (Fig.1.) represents one of the most widely spread freshwater fish across the palearctic region (Bernatchez 2001). Brown trout belong to the class of teleost fish. Their natural habitat is in the upper reaches of water bodies, the so-called trout region, which was eponymous for river descriptions. Characteristic for the trout region, close to the source of a water body, are high oxygen-levels and cold-water temperatures. Brown trout are predatory fish and very territorial (Sterba 2002). At the beginning of the 20th century, fish management changed and the rainbow trout, *Oncorhynchus mykiss*, representing an American salmonid lineage, became more prominent (Crawford 2008, Stankovi et al. 2015).

Figure 1**: Brown trout (*Salmo trutta fario*)**

1.1. Phylogenetic and morphological description of brown trout

Pleistocene glaciations and related climate and environmental changes had a huge impact on the phylogeographic subdivision of brown trout lineages. Five major evolutionary lineages, predominantly allopatric, are known today: *(1) Atlantic, (2) Danubian, (3) Mediterranean, (4) Marmoratus, and (5) Adriatic* (Bernatchez 2001).

S. trutta fario belong to the genus *Salmo* within the family *Salmonidae.* The muscular body and exterior are perfectly adapted and streamlined to get along with the drift (Sterba 2002). Skin color of fish varies individually – some exemplars

do have more or less melanin, especially dorsal in the dorsal area, but the red dots, a distinguishing feature when compared to rainbow trout, are always prominent (Leclercq 2010, Sterba 2002). The adipose fin located on the dorsal side of the body right before the tail fin is common for all *Salmonidae* (Sterba 2002).

1.2. Contributing factors influencing reproduction & decline

Chronological factors define specific time windows for the reproduction of fish species (Juntti and Fernald 2016). Brown trout reproduction occurs from late October until January (Sterba 2002). In this time, brown trout fishing is prohibited by the law of fishery in Bavaria (Bayerisches Fischereigesetz, BayFIG, BayRS 793-1-L, (Art. 1–80)). The side streams of the rivers, with their gravel structures, are the nursery for the eggs and the young of the year trout (Sterba 2002). All *Salmonidae* in common is the genetic sex determination (Quillet et al. 2002, Takashima et al. 1980). Specific sex genes, such as *sdy* (sexually dimorphic on the Y-chromosome), solely expressed by male trout at the beginning of molecular differentiation, are described (Yano et al. 2012, Yano et al. 2013). Environmental factors can influence the development of female or male sex (Kikuchi and Hamaguchi 2013, Juntti and Fernald 2016). Endocrine disruptors, such as environmental estrogen can affect fish embryos. Already this early exposure may limit their reproduction capacity when adult (Schubert et al. 2014). Human medical treatments, such as metformin, which is used for therapy of the civilization disease diabetes type 2, is discussed to reduced reproduction of fish by inducing intersexuality of male fish (Niemuth et al. 2015, Niemuth and Klaper 2015). These ontogenic effects might also affect immunologic competence, e.g. vitellogenin, a specific reproduction protein produced by female fish, has been shown to modulate the immune defense against fish pathogens, like viruses, bacteria or fungi (Zhang et al. 2015). Beside ecotoxicological loads, the advent of a global climate change provides an increased risk for aquatic systems and its inhabitants. Increased water temperature facilitates spread and multiplication of aquatic pathogens, e.g. *Tetracapsuloides bryosalmonae* (causing Proliferative Kidney Disease) or *Aeromonas salmonicida* (causing furunculosis), which is especially critical in habitats with autochthonous species (Carraro et al. 2017, Carraro et al. 2016, Strepparava et al. 2018, Schmidt-Posthaus et al. 2013).

1.3. Anatomy

1.3.1. Liver

The liver assumes responsibility for metabolic processes. A special feature of the liver from fish and cyclostomata is the function as glycogen storage (Hildebrand and Goslow 2004c). In brown trout, the liver has different sizes and shapes, but the gallbladder is always separated, in contrast to common carp. The pancreas is diffusely located between the appendices of the pylorus. (Amlacher 1981).

1.3.2. Spleen

The spleen of fish is able to build every kind of blood cell. Together with the head kidney, the spleen is the prominent blood building organ since fish have no bone marrow like mammalians (Hildebrand and Goslow 2004a, Amlacher 1981). Elimination of dead or defect blood cells is accomplished by erythrophagocytosis (Amlacher 1981). Melanomacrophage centers (MMC) can be found within the spleen (Genten et al. 2009). Just like in other organs (kidney and liver), MMCs have immunological functions (Agius and Roberts 2003).

1.3.3. Kidney

Kidneys of fish manage different osmotic environments, depending on the natural habitat of the species, like fresh water or marine water. Freshwater fish are in need for perfect osmoregulation due to constant hypoosmolar water inflow during food uptake (Hildebrand and Goslow 2004b). The kidney consists of two unseparated parts: The anterior kidney, mainly with blood building function, and the posterior kidney with mainly excretory function. Anterior kidneys mainly consist of hematopoietic tissue and have very few tubular structures. Posterior kidneys consist of glomerula, proximal and distal tubuli and release urine through the ureter (Amlacher 1981).

2. Two main fish diseases contribute to the brown trout decline in pre-alpine rivers of Bavaria

The decline of the brown trout in the limestone pre-alpine rivers of Bavaria is a well-documented phenomenon, which already exists for nearly twenty years. Formerly described by local anglers, massive losses of brown trout during the summer months occurred in Bavarian rivers. The fish showed apathetic behavior and avoided the drift. Respiratory rates increased and fish rapidly died within a few hours. Leading symptoms of the syndrome, the nearly black color of the skin and high mortality rates, reflect the descriptive term Proliferative Darkening Syndrome (PDS) (Schwaiger 2013, Hanfland et al. 2013). The Bavarian Agency of Environment, supported by the Bavarian Fisheries Association (Landesfischereiverband Bayern e.V.), performed broad-scale tests during 2000 and 2009 to investigate whether the syndrome has a chemical-environmental, a toxicological or even an infectious background (Born 2013, Schwaiger 2013). Another infectious disease with similar macroscopically appearance (e.g. black discoloration of the skin), but in contrast to PDS with a well-known etiology, can also be found in Bavaria: Proliferative Kidney Disease, caused by the Myxozoan parasite *Tetracapsuloides bryosalmonae*. Both diseases are influenced by water temperature (Strepparava *et al.* 2018, Schwaiger 2013).

3. Proliferative Darkening Syndrome of brown trout in Bavaria

3.1. Setup and results of exposition trials in 2000 & 2001 – investigation of disease dynamics and species specify

During the first years of controlled exposition trials, disease dynamics and species-specificity were investigated. In 2001, the first state of the art exposition trial was performed in the pre-alpine river Iller near Kempten (Allgäu) (Born 2013).

Wild and hatchery-bred brown trout were kept in long basins supplied with river water from November 2000 until June 2001. Rainbow trout and grayling, not known to be affected by PDS, were kept likewise as reference animals. A brown trout control group was kept in spring water during the whole experiment. Constant examinations of chemical water parameters have been conducted.

Characteristic disease dynamics have been defined as mortalities occurred in all brown trout groups exposed to Iller water in a period from late August until October. Brown trout showed symptoms including dark coloring of the skin, apathy and abnormal swimming behavior. In comparison, brown trout exposed to spring water did not show any clinical signs.

Characteristic pathological lesions during the clinical phase of PDS affected brown trout were identified: Hemorrhages and necrosis of the liver and depletion of splenic white pulp. Posterior kidneys displayed hemorrhages and proliferation of the interstitial tissue. Edema within the stomach wall was found. Gills displayed unspecific telangiectasia. As another distinct pathological feature of PDS affected brown trout displayed progressive anemia and leukopenia. Parasitic examination was negative (Schwaiger 2003).

Grayling and rainbow trout survived the expositions trail without displaying symptoms. Water parameters never passed critical standards for trout during the whole experiment (Born 2013).

3.2. Setup and results of exposition trials in 2002, 2003 and 2004 – distribution of PDS in pre-alpine rivers of Bavaria

To determine the dimension of brown trout mortalities due to PDS in different pre-alpine rivers in Bavaria, exposition trials were reinstalled in 2002 in four selected rivers: Iller, Mangfall, Ammer and Ramsach. Brown trout and rainbow trout were exposed. Hematological examination was done to describe pathological changes within the blood during the whole exhibition time until dying occurred (Schwaiger 2006).

Brown trout of the exposition trial in 2002 displayed characteristic pathological lesions for PDS in liver, spleen, kidney, gastrointestinal tract but also in the gills. Lesions were comparable to preceding examinations of 2001 (Schwaiger 2003, Schwaiger 2006). A major impact of pathological lesions was found in the liver and the spleen. Parasitic infection of single rainbow trout with *Tetracapsuloides bryosalmonae* was found in the Ammer, one PKD positive brown trout was found in the Paar. Kidney lesions, including hyaline droplets within tubular epithelia or interstitial tissue increase, were mainly found in brown trout. Gill lesions with low

impact occurred in all groups, also in rainbow trout control groups (Schwaiger 2006). Shortly before dying, affected brown trout displayed a reduced lymphocyte and erythrocyte population within the blood (Laggerbauer 2003, Schwaiger 2006).

Exposition trials of the following year (2003) were arranged in the pre-alpine rivers Iller, Mangfall and Ammer (Schwaiger 2006). All fish were vaccinated against *Aeromonas salmonicida.* Fish tanks, supplied with Iller water, were equipped with complex filter systems. Three kinds of filter systems were used for potentially pathogen elimination: (1) sand (basalt/quartz), (2) active carbon and (3) UV-radiation (254nm). Measure systems for water parameters (pH, conductivity, temperature, redox-potential, oxygen-saturation) were installed both in fish tanks and in outdoor trials. Parasitological, pathological and hematological examinations were done by the Bavarian Agency of Environment. Residue analyses on two locations should investigate the participation of environmental loads (Schwaiger 2006).

Characteristic pathological lesions for PDS were reproducible in brown trout exposed to Iller water in 2003. Within the filter system fish tank trial, pathological changes, except the diminished glycogen storage, have been lower compared to other trials. Residue analyses provided no evidence of harmful environmental substances. Bacterial and viral examination displayed no infection with specific pathogens. Interestingly, no brown trout of the fish tank trial died due to PDS in contrast to the brown trout exposed in long basins earlier in the year (Schwaiger 2006).

In 2004 the setup was slightly changed as following: Four new locations were implemented for outdoor trials in the Thanners, Isar, Würm and Amper. The stock was already deployed in May 2004. Samples have been delivered to the Friedrich-Loeffer Institute (Insel Riems, Greifwald) for virological examination. Clinical-chemical blood parameters of brown trout were measured (Schwaiger 2006).

PDS occurred quite differently during the exposition trial of 2004: All fish, directly exposed to Iller water died. Sand filter in combination with active carbon reduced the vulnerability of brown trout of being affected with PDS. In aquaria equipped with UV filter, no single brown trout died. In dying groups, pathological lesions

were equal with results of yesteryears trials (Schwaiger 2006). No commonly known salmonid specific bacterial or viral pathogens could be found (Bergmann et al. 2005).

3.3. Setup and results exposition trial of 2005 – temperature dependence of PDS

Main exposition trial of 2005 was conducted at the Iller in a complex experimental setting (Schwaiger 2007). Four groups of brown trout were stocked in long basins supplied with native, cooled (15°C), temporarily UV-treated (from stock until mid-July) and permanent UV-treated Iller water on the 19th of May 2005. In the beginning of the exposition trial, an infection with *Aeromonas spp.* occurred and the fish were treated with Baytril© (Enrofloxacin, 2,5%, 5mg / kg i.p.). Environmental circumstances led to a short break up of the trials, due to a flood in August 2005, and the fish were brought in the hatchery of Wielenbach for an interim time of three days. The fish were held on spring water and returned in their original exposition setup. Pathology samples were taken at week 4, 8, 12, 16, - and 20 after the exposition (Schwaiger 2007).

Permanent UV-treated groups survived the exposition trial without displaying symptoms or typical pathological lesions of PDS supporting the data of 2004. In contrast, all groups of brown trout exposed to native, cooled and temporarily UV-treated Iller water died. Differences in point of time were observed, as mortalities started within the cooled group in early September (09.09.2005) and the native Iller group (11.09.2005). The temporarily UV-treated group displayed the first symptoms in the middle of October (17.10.2005). Pathological lesions were found in all groups, except for the permanent UV-treated and the control group. Rainbow trout were never affected by PDS during the whole trial (Schwaiger 2007).

From late August until September the fish suffered from a heavy lympho- and leucopoenia. All brown trout displayed the already described characteristic histopathological lesions for PDS in the liver, spleen, kidney, gills and gastro-intestinal tract, expect the permanent UV treated group (Schwaiger 2007).

3.4. Setup and results exposition trials of 2008 & 2009 – disease dynamics within distinct river sections, minimal exposition time and different salmonid species

Distribution of the disease within the Iller should be investigated during the exposition period in 2008 (Schwaiger 2013). Different locations were chosen: Kempten, Thanners, Blaichach and Rubi. 300 fish were exposed to native Iller water, lead through aquaria. Additionally, long basins were installed with other trout species such as sea trout (*Salmo trutta trutta*), alsatian char (*Salvelinus alpinus* × *Salvelinus fontinalis)*, brook trout (*Salvelinus fontinalis*) and tiger trout (*Salmo trutta fario x Salvelinus fontinalis*) to investigate, whether other salmonid species can be affected by PDS. The incubation period should be determined more precisely. Therefore, fish were exposed 2, 4, 6, or 8 weeks to native Iller water and afterwards held on spring water. Fish were vaccinated against *Aeromonas* species with AquaVacTM Furovac (0,1 ml / fish) and treated with Baytril© (Enrofloxacin, 2,5%, 5mg / kg i.p.) to exclude that bacterial species have an impact on the disease process.

A manifestation of PDS variates within an affected river system. Locations closer to the alps displayed a decreased dying rate and a time-dependent onset of the mass mortality. A total brown trout loss could be detected at Kempten. Loss in Thanners and Blaichach reached up to 96%. No signs of PDS were found at the location Rubi nearest to the alps, none of the fish died, a mild leucopoenia was found though.

Affection with PDS including mass mortalities in late summer months could be detected in brown trout exposed for 4, 6, and 8 weeks. Within the 2 weeks exposition group, only a small number of fish displayed symptoms of Proliferative Darkening Syndrome.

Hybrids of salmonid species displayed similar symptoms compared to brown trout affected by PDS. Time windows and extent of mortalities varied between hybrids, close related salmonids and brown trout. Mass mortalities started at first in tiger trout at the beginning of September and reached 100 %. In purebred fish, such as brook trout or alsatian char, mortalities started mid-September. Mortalities in

both species were up to 41 % compared to brown trout. Last point of time for observed mortalities in alsatian char was in December 2008. This trial allowed a deeper insight in different kinds of affection by PDS in closely related species of brown trout (Schwaiger 2013).

The setup in 2009 was done at the Iller and the Premer Mühlbach (Schwaiger 2013). Fish were exposed for two weeks (single group) and five weeks in Iller water. Exposure at Premer Mühlbach was done for eight weeks, as well as one permanent group. All groups were held in fish tanks supplied with spring water after fulfilled exposition time until the first symptoms of PDS were visible. Temperature experiments were done as some fish tanks were raised to imitate conditions within the Iller and one was cooled down to 10°C. Not permanent exposed fish of Premer Mühlbach were also held in a temperature aquarium. Transmission trials between brown trout and rainbow trout were performed. Brown trout survivor of 2008 were exposed again (Schwaiger 2013).

PDS was seen in 5 weeks exposed groups of 2009. Comparable to 2008, a 2 weeks exposition was not enough for infection. Transmission trial of 2009 failed, as there was no transmission of PDS between exposed brown trout held with control brown trout and rainbow trout. Rainbow trout never displayed symptoms of PDS such as seen in yesteryears trials. Re-exposed survivors of 2008 showed little immunity against PDS, as 35 % of these group survived the exposition trials of 2009 (Schwaiger 2013).

3.5. Summary of the exposition trials

The first exposition trial in water of the pre-alpine river Iller indicated the narrow time range of occurring mortalities, the single affected species brown trout and the macroscopically appearance of the syndrome (Born 2013). Summarizing the results, exposition trials during 2002-2004 pointed out the reproduceable, progressive pathological and hematological changes during a small-time window before dying. Time of exposition plays a major role for PDS, as fish exposed later than July displayed no dying in late August and no characteristic pathological lesions. Mortalities can be prevented with UV-filtration. Combination of sand and active carbon filters reduce intensity of PDS but do not prevent the infection. Already known fish pathogens were not detected in all trials. Therefore, PDS seems to be a time-related disease with a long incubation period until final disease stadium. As living material can be eliminated by UV, an infectious cause is conceivable based upon earlier results. Temporary UV-treatment of Iller water does not prevent the infection, but the progression of the disease is slower, and clinical symptoms start nearly five weeks after dying compared to native and cooled groups. Dying also occurred within the cooled group, even a little later compared to the native group. All in all, an infectious disease seems possible (Schwaiger 2006).

In a feeding trial performed by the Friedrich-Loeffler-Institute in 2006, PDS was induced in formerly healthy brown trout, which fed on diseased organ material. Although the fish were not exposed to Iller water, clinical symptoms were similar to PDS in the Iller (Köllner 2006). In contrast to this feeding experiment, a direct transmission tested in a cohabitation trial was not successful. A minimal incubation time of four weeks in Iller water can lead to reproduceable clinical symptoms and mass mortalities (Schwaiger 2013).

4. Piscine Orthoreovirus 3 – a fish pathogen mistakenly associated with PDS

Piscine Reovirus, (Genus *Orthoreoviridae*, Subfamily *Spinaviridae*) is widely spread and can be found in farmed and wild trout populations. Current studies underline presence also in Germany (Adamek et al. 2019, Garseth et al. 2013, Fux et al. 2019). Reoviridae are double stranded RNA viruses with ten linear genome segments: L1-3, M1-3 and S1-4. Three subtypes of PRV are known worldwide – PRV1 mainly in farmed Atlantic Salmon (*Salmo salar* L.), especially in Norway, PRV2 only known from Japan and PRV3, recently described in Norway, Denmark, Chile and Germany (Takano et al. 2016, Kibenge et al. 2013, Adamek *et al.* 2019, Dhamotharan et al. 2018). PRV1, firstly described in 2004, is associated with the Heart and Skeletal Muscle Inflammatory Disease (HSMI) of Atlantic Salmon. Histopathological lesions occur within heart and skeletal muscles. PRV1 causes high morbidity but mortality rates are low. HSMI can be transmitted by cohabitation as well as intraperitoneal injection of infectious material (Kongtorp and Taksdal 2009, Kongtorp et al. 2004). The Erythrocytic Inclusion Body Syndrome (EIBS) is caused by PRV2. Mortalities in Coho Salmon (*Oncorhynchus kisutch*) and Chinook Salmon (*Oncorhynchus tshawytscha*) are described. Virions can be found as inclusion bodies in cytoplasm of erythrocytes, which leads to massive anemia due to replication. Necroses within heart muscles fibers are possible (Takano *et al.* 2016). In 2017, PRV3 was firstly detected in Germany. Clinical signs occurred in rainbow trout breeding farms. Fish displayed apathy and lethargy; mortality rates were up to 20% (Adamek *et al.* 2019). In 2018, a piscine reovirus was erroneously described as causative agent of PDS in brown trout mortalities in Bavaria (Kuehn et al. 2018). In a follow-up study, a direct association of this PRV3 with PDS could not be confirmed. Viral nucleic acid, detected by qPCR, was also found in healthy control brown trout. Fish with confirmed PDS were PRV3 nucleic acid free (Fux *et al.* 2019). Thus, the causative agent of PDS remains still unclear.

5. Proliferative Kidney Disease (*Tetracapsuloides bryosalmonae)*

5.1. Characteristics and distribution of *T. bryosalmonae*

Tetracapsuloides bryosalmonae belongs to the class Malacospora, phylum Myxozoa (Canning et al. 2000, Canning and Okamura 2004). Myxozoa include more than 1.200 species in their clade, mostly with an endoparasitic life cycle (Anderson et al. 1998). The myxozoan species cause high mortality in fish farms worldwide, including *Myxobolus cerebralis* and *T. bryosalmonae* (Ferguson 1979, Clifton-Hadley 1984, Henderson and Okamura 2004, Skovgaard and Buchmann 2012). Rainbow trout, brown trout, brook trout, grayling and pike are known for their susceptibility to PKD (Seagrave 1981, Feist and Bucke 1993). Parasitic structures, found in histology, were named PKX in the beginning of the eighties (Seagrave 1980). This myxozoan endoparasite species is characterized by multiple spore stages and formation of polar capsules (Morris and Adams 2008).

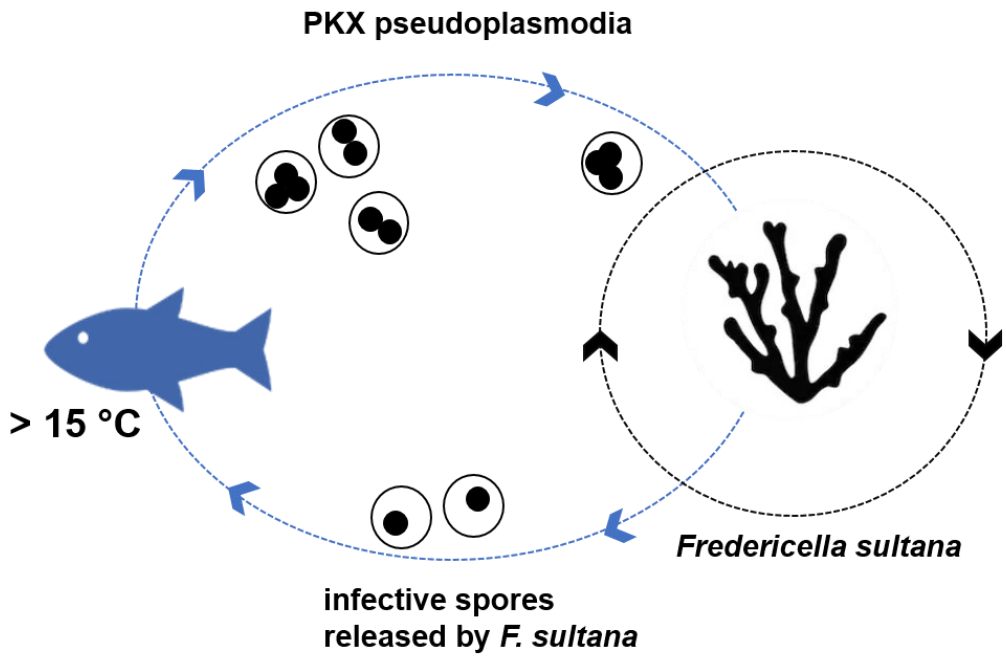
North American and European strains from *T. bryosalmonae* are known. Phylogeographical analyses displayed, that *T. bryosalmonae* strains in Europe are less genetic divergent than strains in the United States. Nevertheless, European strains of *T. bryosalmonae* can induce PKD in rainbow trout (Henderson and Okamura 2004). Aquaculture is likely not the origin of the PKD-spread over Europe, as the internal transcribed spacer 1 (ITS-1) sequences examined from different European locations are highly variable in comparison with the strain from the U.S. and are quite diverse among themselves. In contrast, in strains from *M. cerebralis*, which are known to spread via aquaculture and trading, ITS-1 sequences are nearly identical (Henderson and Okamura 2004). Geographical spread of *T. bryosalmonae* may be further explained by overcoming long distances by transport of infectious material, like statoblasts, via waterfowl or even ballast water of ships (Abd-Elfattah 2013, Figuerola et al. 2004, Okamura 2011, Sudhagar et al. 2019). Efficiency of the parasite is given through its complex but perfectly established life cycle in two hosts (Carraro *et al.* 2016). Beside adaption processes within the life cycle, temperature is a key feature in the development of *T. bryosalmonae* (Schmidt-Posthaus et al. 2012, Bettge et al. 2009). Climate change

and rising water temperatures encourage the invasion of PKD within European rivers (Carraro *et al.* 2016).

5.2. Life cycle of *T. bryosalmonae*

T. bryosalmonae develops in a two-host lifecycle. Thereby, freshwater bryozoa, such as *Fredericella sultana,* function as non-vertebrate host for the causative infectious cell which further leads to disease in the vertebrate host – the fish (Anderson et al. 1999, Tops and Okamura 2003, Feist et al. 2001). Invasion and timing of clinical PKD can be explained by the ubiquity of bryozoa and their growth in late spring/ early summer. Bryozoa are highly tolerant against changes in their environment, such as changing pH sets, and they can be found in still water as well as in the drift (Anderson *et al.* 1999).

Figure 2: **Two host life cycle of *Tetracapsuloides bryosalmonae***



Infection of river sites occurs from both sides: Fish and bryozoa (Tops and Okamura 2003, Abd-Elfattah et al. 2014, Morris and Adams 2006). Parasitic development within the metacoel of *F. sultana* leads to covert and overt infection of bryozoa. Vertical transmission is possible. Maternal colonies are able to produce infective statoblasts by being covert or uninfected with PKD (Abd-Elfattah 2013). Fish, infected with *T. bryosalmonae* two years ago, are still able to cause infection in bryozoan colonies (Abd-Elfattah *et al.* 2014).

T. bryosalmonae forms sacs within the body cavity of the bryozoan host. The spherical spore sacs contain pre-sporogonic or sporogonic stages of the parasite (Canning *et al.* 2000, Mc Gurk et al. 2006). Parasitic development and sporogony are closely related to the sac wall. Sporogonic stages of *T. bryosalmonae* are formed by nuclear division. Polar capsules, typically for myxozoan species, are formed during sporogony (Canning *et al.* 2000, Kent and Hedrick 1985).

The infective dose of parasitic spores transmitted from wild *F. sultana* colonies to rainbow trout was examined by Mc Gurk and colleagues (2006). Bryozoan colonies were collected from a river known to be endemic for PKD. The life cycle within the bryozoan is finished within a few days until infective spore material is released by sac rupture. *T. bryosalmonae* reproduces effectively, as a small amount of five zooids ended up in thousands of infective spores. Respectively, exposition of uninfected rainbow trout to a single spore can lead to PKD (Mc Gurk *et al.* 2006).

Morris described two main stages of parasite development stages within the interstitial tissue of the kidney: Primary cells and cell-doublets, a primary cell with an engulfed secondary cell. Pseudoplasmodia, the only form allowed to pass the tubule lamina, develop in complex cell division of these interstitial forms. Essential are two capsulogenic cells, which form the further polar capsules (Morris and Adams 2008).

Spores, released via the urine of trout, infect new bryozoan colonies (Grabner and El-Matbouli 2008, Hedrick et al. 2004). Infection progress varies between salmonid species, as intratubular sporogonic stages had been detected in high amounts in tubules of brown trout, whereas the pre sporogonic stages were low in liver and spleen. In comparison, rainbow trout showed no sporogonic stages in kidneys but high amounts of pre sporogonic stages in liver and spleen (Kumar et al. 2013). Recent studies described persistence of *T. bryosalmonae* within brown trout for at least five years (Soliman et al. 2018).

Diversity of *T. bryosalmonae* parasitic strains have been discussed as a reason for adaption and the further development within the fish host (Henderson and Okamura 2004, Bucke 1991). A possible explanation for higher losses of rainbow trout due to infection with the European strain of *T. bryosalmonae* may be less

adaption compared to native brown trout (Grabner and El-Matbouli 2008).

PKD is a temperature-related disease and clinical signs and mortality increase above 15 °C (Clifton-Hadley 1984, Ferguson 1981, Bettge *et al.* 2009). Fish surviving the clinical phase of PKD infection can recover. Elimination of *T. bryosalmonae* during recovery is temperature independent but seems to occur at high as well as low water temperatures (Schmidt-Posthaus *et al.* 2012). In rainbow trout, infection with PKD is possible during the whole year (Gay et al. 2001).

5.3. Pathology caused by *T. bryosalmonae*

Infection can occur via gills (Morris et al. 2000). Another study suggests that the site of infection may additionally be the skin due to the lack of pathological changes in the gills after infection (Feist *et al.* 2001). The parasite also infects liver and spleen, which can be explained through its spread via blood (Clifton-Hadley 1987a, Kent and Hedrick 1985).

Clinical signs of PKD are exophthalmos, darkening of the skin – a rather unspecific symptom (Leclercq 2010, Kittilsen et al. 2009) – and anemia, visible as pale gills (Hedrick 1993, Clifton-Hadley 1987b). Swelling of the kidney can occur with more or less lesions – the Clifton-Hadley Index can be used for macroscopic scoring of the kidney swelling. Histological findings contain vascular, tubular and mostly interstitial changes (Clifton-Hadley 1987b, Clifton-Hadley 1984). PKD leads to massive granulomatous infiltration and proliferation of the hematopoietic tissue, mainly in the posterior kidney. Hemorrhage and necrosis are also described. In some cases, vasculitis was seen (Schmidt-Posthaus *et al.* 2013). Other studies described temperature-related and time-related kidney lesions (Bettge *et al.* 2009). Fish surviving a clinical PKD infection develop protective immunity (Foott and Hedrick 1987).

5.4. Impact of *T. bryosalmonae* on the immune system of infected fish

The innate immune system of fish reacts during clinical PKD infection with renal hyperplasia based on proliferation processes of the pronephron lymphocytes. In contrast, other cell populations of the hematopoietic tissue remain stable. In situ analysis showed an increase of S and G2/M phase of the cell cycle of leucocytes, which is markable for cell growth. Macrophage activity and oxidative burst are

reduced within a clinical PKD infection. Granulocytes are underrepresented (Chilmonczyk 2002).

Holland and colleagues (2003) studied gene expression profiles of PKD-infected rainbow trout, particularly with regard to cytokines (TNF-α2) and the inflammatory response enzyme COX-2. They observed initial upregulation of TNF-α2 and COX-2 is followed by downregulation of both during the clinical phase of PKD. Additionally, a lack of upregulation of IL-8, attracting further macrophages and T-cells (acquired immune system) during a parasite infection, was identified. A pathogen-dependent pattern within the gene expression profile and participating dominant immune cells seem to be conceivable, as not the inflammatory response of leucocytes is dominant but the abnormal proliferation (Holland *et al.* 2003). Nevertheless, inflammatory responses, as seen in brown trout, are uncommon for infections with myxozoan species (Anderson *et al.* 1999).

PKD can lead to mild up to severe infection in salmonids. The most underestimated value of the disease is the strong immunosuppression of the fish, mainly described for rainbow trout This leads to an increased vulnerability for additional secondary infections, especially under field conditions (Hedrick 1993, Chilmonczyk 2002, Holland *et al.* 2003).

5.5. Infection with *T. bryosalmonae* linked to decline of brown trout in European rivers

Previous studies estimated the decline of wild brown trout populations affected by PKD in Estonia, Denmark, Switzerland and Austria (Wahli et al. 2008, Skovgaard and Buchmann 2012, Dash and Vasemagi 2014, Gorgolione 2016). In 2016, PKD was associated to play an important role in the decline of brown trout in Austria and may be an underestimated part in the etiology of Proliferative Darkening Syndrome (Gorgolione 2016). A recent study, summarizing all aspects of PKD-infection, supported the hypothesis of protracted decline of wild brown trout populations due to PKD with often quite unnoticed mortalities (Sudhagar *et al.* 2019).

III. OBJECTIVES

In terms of high mortalities within wild brown trout population in the pre-alpine Isar river, a participation of Proliferative Kidney Diseases and Proliferative Darkening Syndrome in brown trout was investigated. The following aspects were addressed:

i. Examination of moribund brown trout in the pre-alpine Isar river with pathological, immunohistochemical and biomolecular assays

ii. Participation of Proliferative Darkening Syndrome in moribund Isar brown trout
   - Histological examination of liver and spleen tissue samples
   - Rule out of Piscine Orthoreovirus 3 infection through qPCR

iii. Gross pathology displayed renal lesions and infection with *T. bryosalmonae*
   - Histological examination of anterior and posterior kidney samples
   - Implementation of an immunohistochemical assay for parasitic detection
   - Implementation of qPCR for detection of parasitic DNA

iv. Quantitative stereological analyses of brown trout posterior kidneys affected by *T. bryosalmonae*

v. Phylogenetical analyses of the parasitic *T. bryosalmonae* strain found in affected Isar brown trout

IV. RESULTS

The manuscript is presented in form accepted for publication (Arndt et al., 2019) in *Pathogens (MDPI).*

Proliferative Kidney Disease and Proliferative Darkening Syndrome are Linked with Brown Trout (*Salmo trutta fario*) Mortalities in the Pre-Alpine Isar River

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For many years, brown trout (*Salmo trutta fario*) mortalities within the pre-alpine Isar River in Germany were reported by the Bavarian Fisheries Association (Landesfischereiverband Bayern e.V.) and local recreational anglers during August and September. Moribund fish seemed to be affected by proliferative darkening syndrome (PDS). In addition, proliferative kidney disease (PKD) caused by *Tetracapsuloides bryosalmonae* was discussed. To investigate this phenomenon, the present field study monitored brown trout mortalities by daily river inspection in 2017 and 2018. Moribund brown trout (n = 31) were collected and examined using histology, immunohistochemistry, qPCR, and quantitative stereology. Our investigations identified 29 (93.5%) brown trout affected by PKD. Four brown trout (12.9%) displayed combined hepatic and splenic lesions fitting the pathology of PDS. The piscine orthoreovirus 3, suspected as causative agent of PDS, was not detectable in any of the samples. Quantitative stereological analysis of the kidneys revealed a significant increase of the renal tissue volumes with interstitial inflammation and hematopoietic hyperplasia in PKD-affected fish as compared to healthy brown trout. The identified T*. bryosalmonae* strain was classified as part of the North American clade by phylogenetical analysis. This study highlights PKD and PDS as contributing factors to recurrent autumnal brown trout mortalities.

Introduction

In the last decade, high mortalities and a dramatic decline of the brown trout populations were reported by the Bavarian Fisheries Association (Landesfischereiverband Bayern e.V.) and local recreational anglers in the pre-alpine Isar River in Southern Bavaria (Germany). These mortalities were predominantly observed in the warmer summer months. Facing the advent of global climate change, aquatic ecosystems are particularly endangered because increasing water temperature is an important factor for aquatic pathogen spread and multiplication (Carraro *et al.* 2016, Strepparava *et al.* 2018). Autochthonous species, specialized in colonization of specific habitats, are especially vulnerable to changes of their microenvironment (Alborali 2006). In the Isar River, the decline seems to solely affect brown trout (*Salmo trutta fario*). Thus, adult brown trout, capable of reproduction, were regularly restocked in the past years in

order to regenerate and stabilize the natural populations. Still, stocking failed to halt the decline of brown trout. Adult brown trout capable of reproduction were mainly found moribund or already dead during August and September. The scale of the decline is probably high, but precise data is missing because declined brown trout populations are hard to monitor. Anglers described apathetic behaviour and black discoloration of moribund brown trout. These rather unspecific symptoms have been described for various different fish diseases (Amlacher 1981). However, these clinical signs also match two disorders affecting brown trout from other rivers in the Alpine region: proliferative darkening syndrome (PDS) and proliferative kidney disease (PKD) (Hedrick 1993, Schwaiger 2013). So far, none of these diseases have yet been reported to occur in the Isar River and various other pre-alpine rivers in Bavaria.

For decades, PDS has caused a massive decline of brown trout populations in the grayling zone of several pre-alpine rivers in Southern Germany (Schwaiger 2013, Lahnsteiner et al. 2009). These mass mortalities affect a major part of the brown trout population every late summer until autumn, whereas rainbow trout (*Oncorhynchus mykiss*) populations are not affected. The specific cause of PDS is unknown and potentially multifactorial. Piscine orthoreovirus 3 (PRV-3) has been recently suggested as the potential cause of PDS (Kuehn *et al.* 2018), but this hypothesis could not be confirmed and rather PRV-3 seems to cause a subclinical bystander infection (Fux *et al.* 2019). Despite various investigations within the last decade, a causative agent has not been detected yet (Schwaiger 2013, Kuehn *et al.* 2018). Therefore, the diagnosis of PDS is solely based on epidemiological data (seasonal occurrence), macroscopic lesions (darkening of skin), and histological lesions (liver necrosis in combination with depletion of splenic white pulp) (Fux *et al.* 2019). PKD has recently been suggested as a potential cofactor in PDS (Gorgolione 2016). The responsible parasite *Tetracapsuloides bryosalmonae* already represents a relevant pathogen for trout in Austrian rivers (Gorgolione 2016).

PKD is a parasitic disease in farmed and wild rainbow and brown trout with a strong immunosuppressive character and the capacity to cause high mortalities which particularly occur in the northern hemisphere (Hedrick 1993, Clifton-

Hadley 1984, Ferguson 1979, Holland *et al.* 2003). Besides darkening of the skin, PKD-affected fish mainly show swelling of the kidneys and anemia (Hoffmann 1984, Clifton-Hadley 1987b), pale yellow livers are also noticed (Clifton-Hadley 1987b).

The causative myxozoan, *Tetracapsuloides bryosalmonae*, has a complex two-host lifecycle within freshwater bryozoa, mostly *Fredericella sultana* (Tops and Okamura 2003, Feist *et al.* 2001) Freshwater fish are infected through gills or skin (Grabner and El-Matbouli 2008, Morris 2000). The parasite migrates through blood vessels into its main target organ, the kidney (Feist *et al.* 2001, Clifton-Hadley 1987a, Kent and Hedrick 1985). In the kidney, the parasite is located within the interstitium and the tubules causing interstitial inflammation and hematopoietic proliferation (Hedrick 1993, Clifton-Hadley 1987b). After renal development of the parasite, spores are produced and released via urine to start a new lifecycle (Kent and Hedrick 1985, Hedrick *et al.* 2004, Soliman *et al.* 2018). In severe courses of the disease, hematopoietic hyperplasia and inflammation in the kidney result in impairment of renal function (Clifton-Hadley 1987b, Schmidt-Posthaus *et al.* 2013). PKD also leads to immunosuppression by dysregulation of the cellular immunity making the infected fish more susceptible to secondary infections (Holland *et al.* 2003).

Water temperature plays an important role in the pathology of PKD-infected fish. Clinical signs and mortality are significantly increased above 15 °C (Schmidt-Posthaus *et al.* 2012, Bettge *et al.* 2009). Mortality rates in wild fish are usually less than 20%, however in the presence of secondary infections mortality rates are probably higher (Hedrick 1993, Hoffmann 1984, Mc Gurk *et al.* 2006). The brown trout decline, partially caused by PDS, is suspected to occur in a large body of pre-alpine rivers and its effect on brown trout populations is probably massive (Schwaiger 2013). However, neither presence nor prevalence of various piscine pathogens have been characterized in detail for most southern German rivers. This is also true for the third largest river in Bavaria, the Isar. The apparent brown trout decline warrants qualitative and quantitative investigations to highlight potential causes, to provide a basis for epidemiological investigations and the identification of countermeasures and to improve the knowledge on

interrelated disease processes. Thus, the present study was initiated by the Bavarian Fisheries Association (Landesfischereiverband Bayern e.V.), with local anglers reporting annual recurring brown trout mortalities in the Isar River. In this field study, we investigate specimens of the brown trout mortalities in 2017 and 2018 using state-of-the-art pathological and molecular biological analyses. Lesion distribution and quality were pathologically assessed. Parasite burdens and their impact on inflammatory lesions and tissue damage in diseased brown trout were determined with qPCR analyses and unbiased quantitative stereology. Our results demonstrate that PKD and PDS contribute to the recurrent mortalities of Isar brown trout.

Results

Brown Trout Mortalities Occur at Specific Isar River Sections within Munich

In 2017, moribund brown trout were only observed at specific sites in the main stream of the Isar River. In 2018, most fish were observed in the meadows, in a smaller side stream called Auer Mühlbach (Figure 1).

During the observation period, multiple dead but relatively few moribund brown trout were observed in both years. In total, 24 moribund brown trout were sampled in 2017 and seven in 2018. Apparent mortalities in other fish species were not observed. Affected brown trout were apathetic and swimming velocity and general activity were greatly reduced. The fish avoided the water current, stood near the shore, and lost their natural flight reaction. Collected brown trout had a mean size of 35.5 cm (standard deviation [SD] = 4.5) in 2017 and 28.6 cm (SD = 4.6) in 2018. Female and male brown trout were equally affected (19 and 12 animals, respectively). The water temperature during this time period was not unusually high when compared to the preceding years (Figure 2).

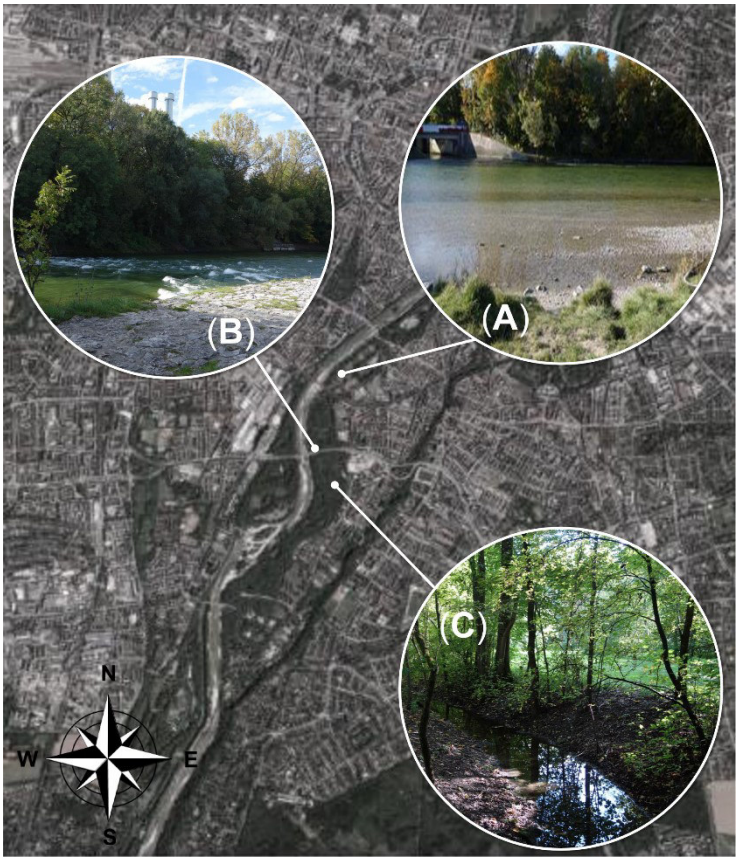

Figure 1. Sampling sites at the Isar River, Munich: (**A, B**) 2017 hotspots, main stream of the river (**A**: 48°07'06.7"N 11°33'40.3"E , 48°07'10.1"N 11°33'43.0"E; **B**: 48°06'44.3"N 11°33'34.3"E); (**C**) 2018 hotspot, Isar meadows, Auer Mühlbach (48°06'45.3"N 11°33'46.5"E).

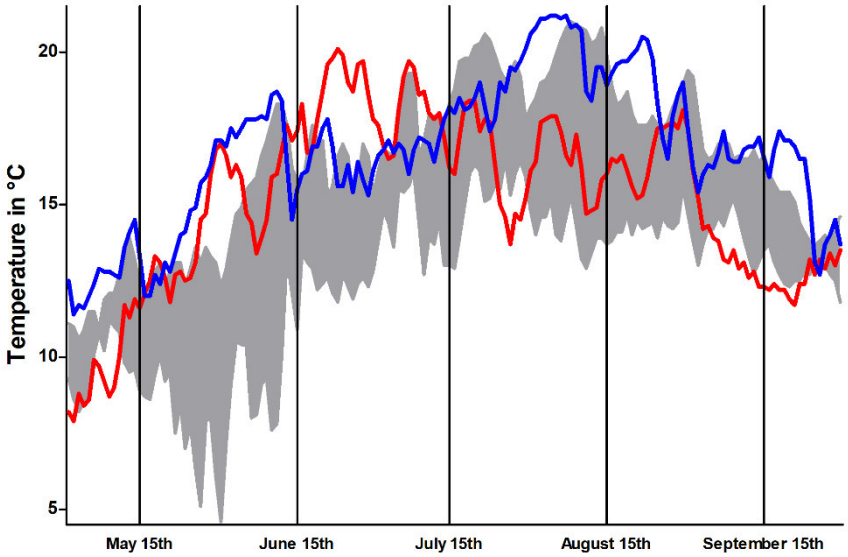

Figure 2. Temperature profile, representing mean daily temperature within the Isar River during sampling periods in 2017 (red) and 2018 (blue), compared with temperature range of the preceding four years (grey area). Data from the Bavarian Agency of Environment (https://www.gkd.bayern.de).

Necropsied Brown Trout Mainly Displayed Renal and Hepatic Lesions

All sampled trout underwent pathological examination. The skin of the sampled moribund brown trout was dark and showed a nearly black color (Figure 3). Many brown trout displayed spread opercula before necropsy as a sign of increased respiration. The gills were pale (indicative of anemia) and in one case hemorrhagic. Routinely performed native gill and skin smears were free of microscopically detectable ectoparasites. Exophthalmos was present in 10/31 (32%) of brown trout. One fish showed bilateral corneal opacity. Livers and spleens were mildly enlarged. The anterior and posterior kidneys were swollen with dark red color and a micronodular surface. Body fat stores were considerably reduced, indicating poor nutritional status.

Figure 3. Affected brown trout with black skin color.

Bacteriologic culture of heart, liver, spleen, and posterior kidney resulted in bacterial growth in 16/31 (52%) fish. MALDI-TOF mass spectroscopy of these cultures led to the identification of *Aeromonas salmonicida*, the etiological agent of furunculosis, in the posterior kidney of one fish. However, skin and inner organs of this brown trout displayed no macroscopic or histologic lesions compatible with furunculosis. The mass spectra of the other bacteria did not coincide with commonly known fish pathogens and histology did not reveal bacterial infection. Of note, the causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum*, could not be identified in cultures from the posterior kidneys and kidney histology did not indicate bacterial infection.

Brown Trout Displayed Lesions Compatible with PKD and PDS

To complement necropsy results, brown trout organs were assessed via histology. Histologic lesions compatible with PDS (defined as combined splenic and hepatic lesions) were detected in 4/31 brown trout (13%). Solitary splenic or

hepatic lesions were identified in 27/31 (87%) fish. All individuals with lesions indicating PDS also displayed kidney lesions typical for PKD. In total, kidney lesions compatible with PKD were detected in 29/31 (94%) sampled brown trout.

The examined livers showed loss of hepatocyte vacuolation in every specimen suggesting diminished hepatic glycogen storage. Additionally, 7/31 (23%) brown trout displayed multifocal random liquefactive hepatic necrosis (Figure 4). Ten of 31 livers were severely congested and many displayed few perivascular and periportal lymphocytes and histiocytes. In 27/31 (87%) spleens, the lymphocytes of the white pulp were depleted (Figure 4) and red pulp areas congested. Seven of 31 (23%) brown trout displayed gastrointestinal submucosal edema. Liver samples tested by RT-qPCR for PRV-3-specific RNA were negative in all of the brown trout sampled in 2017 and 2018.

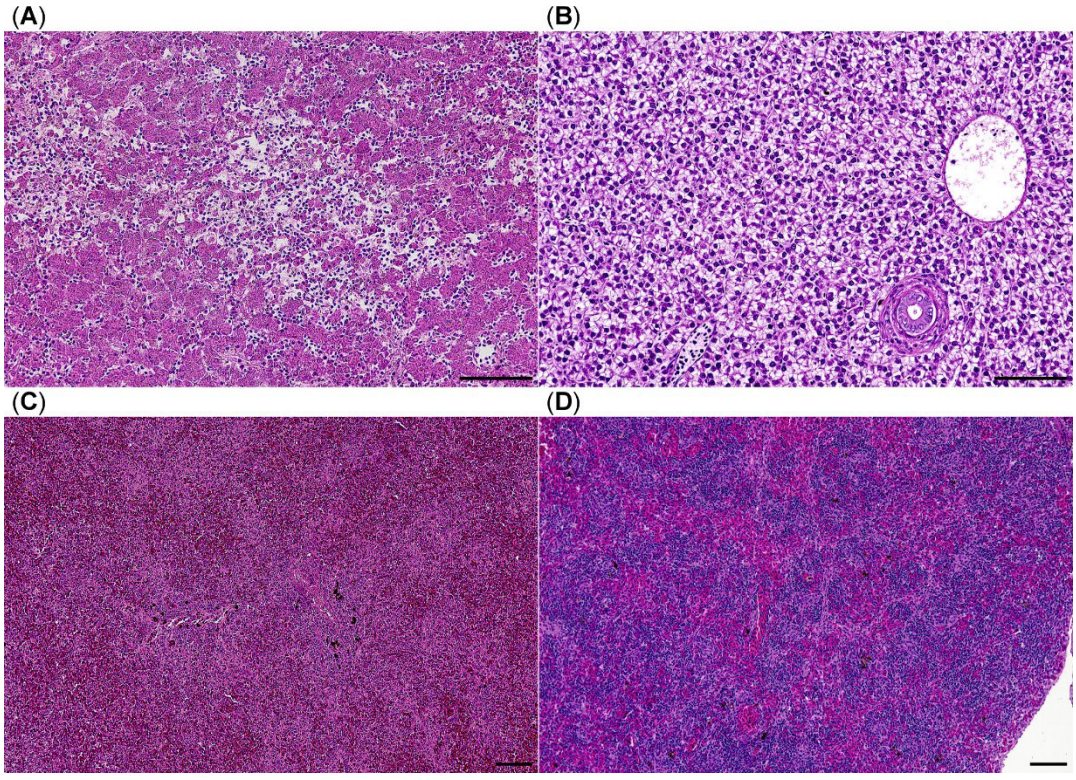

Figure 4. Histology of brown trout liver and spleen, HE stain, (**A**) liver with multifocal liquefactive necrosis (center) and viable hepatocytes without vacuolation suggesting depletion of glycogen stores; (**B**) liver from a healthy brown trout; **(C)** congested spleen with lymphocytic depletion of white pulp areas; **(D)** spleen from a healthy brown trout; bars = 100 µm.

Anterior and posterior kidneys showed mild to moderate interstitial nephritis consisting of an infiltration with lymphocytes and histiocytes. Interstitial hematopoietic tissue was increased as well. The branches of melanomacrophage centers were disrupted due to the increased inflammation and hematopoiesis

(Figure 5). Several tubular and interstitial parasitic pseudoplasmodia compatible with *T. bryosalmonae* were detected already in routine stains. To detect intralesional parasites with higher sensitivity, an anti-*T. bryosalmonae*-immunohistochemistry was implemented. In 14/31 (45%) brown trout posterior kidneys interstitial and tubular *T. bryosalmonae* pseudoplasmodia were immunohistochemically detected (Figure 5).

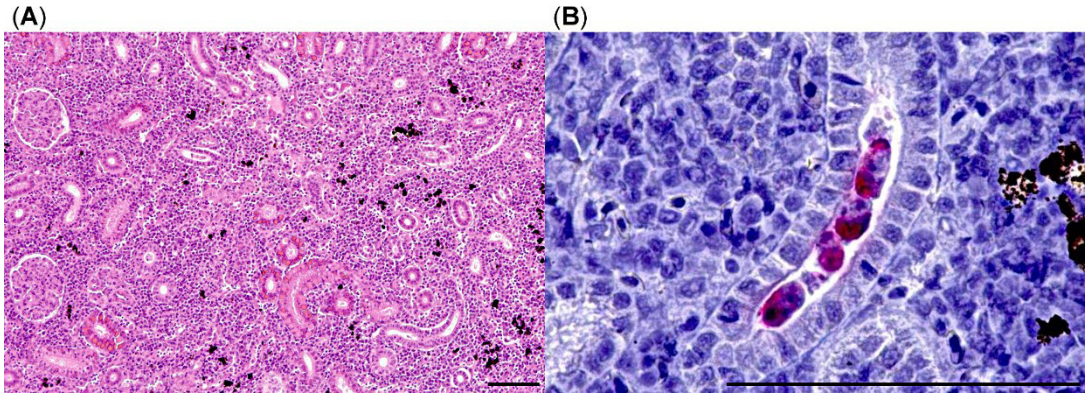

Figure 5. Histology of brown trout posterior kidney, **(A)** increased interstitial cellularity with separation of melanomacrophage centers, HE stain; **(B)** multiple tubular parasitic pseudoplasmodia (in red), anti-*T. bryosalmonae*-immunohistochemistry; bars = 100 µm.

The Majority of Brown Trout Kidneys Were Positive for T. bryosalmonae DNA

Although immunohistochemistry did confirm *T. bryosalmonae* infection in 14/31 (45%) fish, macroscopic kidney lesions and routine histology suggested a higher rate of infection. Thus, we performed an additional qPCR to detect *T. bryosalmonae* with higher sensitivity and to compare parasite load. In total, 29/31 (94%) kidney samples were determined to be positive by qPCR. High DNA-levels were detected within the kidney of 6/24 brown trout in 2017 with ΔCq-values over 22, whereas the majority had ΔCq-values in the range of 15–22. In 2018, all sampled fish showed ΔCq-values between 20–23 (Figure 6). The alignment with 18S reference sequences (FJ981823, KF731712, KJ150286, KJ150287, KJ150288) proved a 99–100% identity to the *T. bryosalmonae* genome.

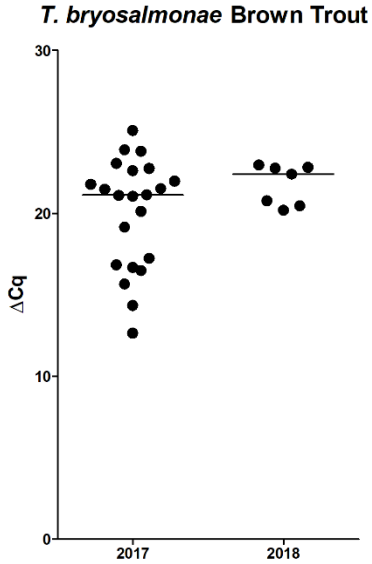

Figure 6. qPCR for *T. bryosalmonae,* of brown trout posterior kidneys, ΔCq values (2017: 22/24 positive; 2018: 7/7 positive); bars represent medians.

For phylogenetical analysis we used a genome fragment extending from 18S through the internal transcribed spacer 1 (ITS-1) and terminating in the 5.8S region. The PKD strain found in the Isar River could be assigned to the North American Clade [29] of *T. bryosalmonae* (Figure 7).

To monitor PKD presence in healthy trout, we decided to use non-affected brown trout caught by fly fishery before and after reported mortalities occured. During fishing season in 2018 (May–October), selected local anglers were asked to provide kidney samples from caught brown trout. However, neither the authors nor the participating anglers did catch a single brown trout, only rainbow trout were captured (n = 50). Although brown trout were not captured, we still examined the rainbow trout posterior kidneys as a surrogate to assess PKD presence. In the qPCR analysis, 33/50 of rainbow trout posterior kidney samples were PKD positive. The distribution of ΔCq values of the positive rainbow trout was comparable to the range detected in the brown trout (Figure 8).

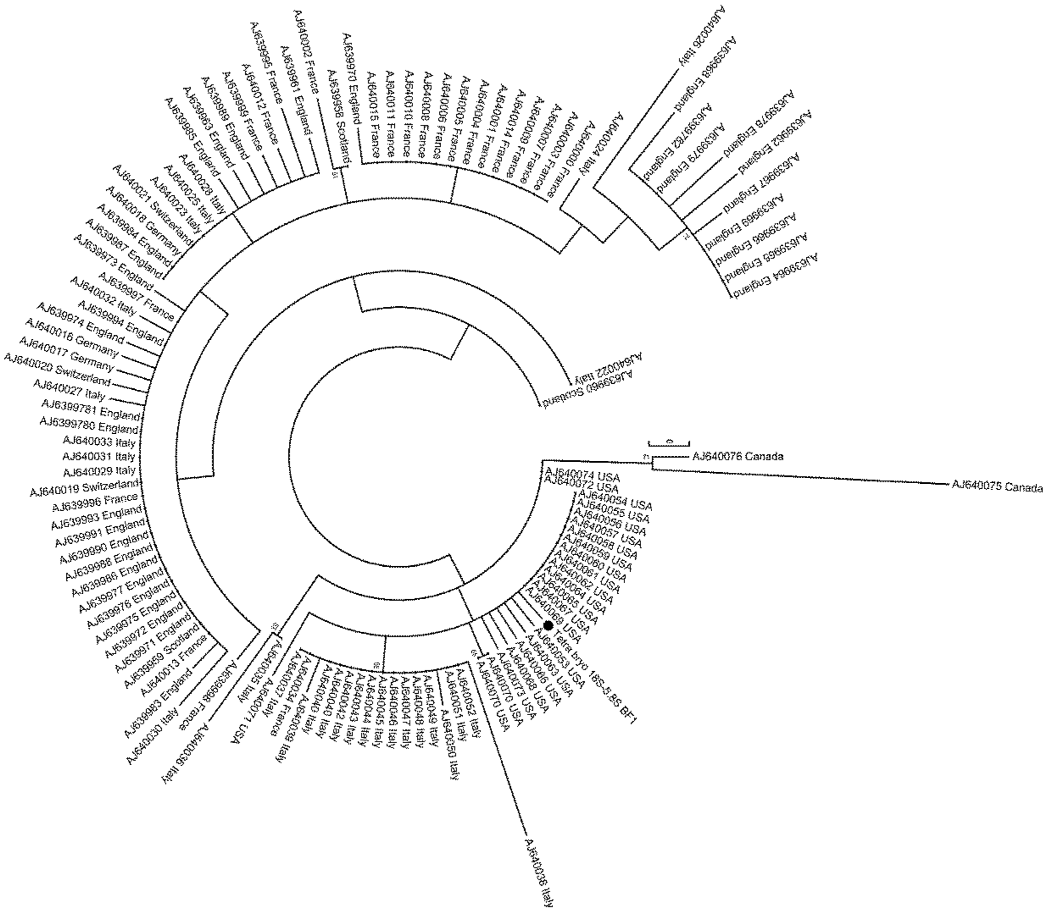

Figure 7. Phylogenetic analysis of *T. bryosalmonae*, including the Isar strain (black dot) grouped into the North American clade of *T. bryosalmonae*.

In summary, histology and qPCR showed a high prevalence of PKD within all sampled trout. As expected, immunohistochemistry showed a lower sensitivity when compared to qPCR.

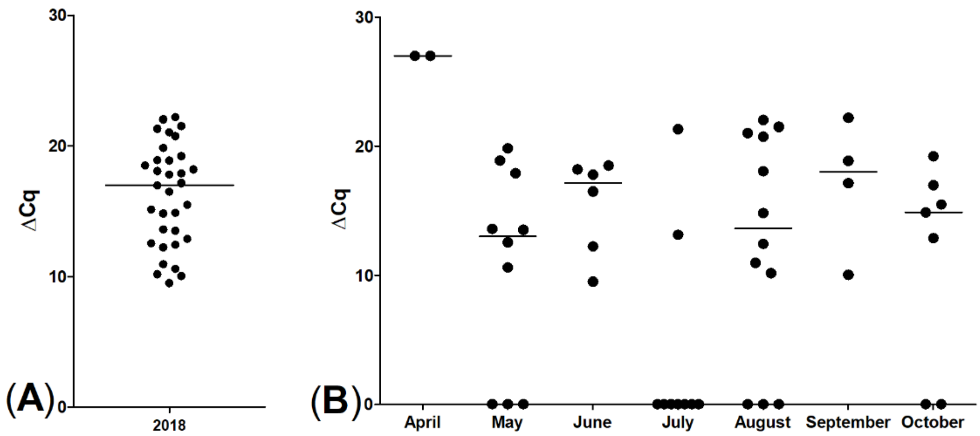

Figure 8. qPCR for *T. bryosalmonae* of rainbow trout posterior kidneys, ΔCq values, (**A**) 33/50 positive; (**B**) sampling months of all rainbow trout; displaying spread of proliferative kidney disease (PKD) infection during the whole fishing season, bars represent medians.

Impact of PKD on Renal Inflammation and Tissue Remodelling in Brown Trout

To advance the characterization of the detected kidney lesions, which were associated with the *T. bryosalmonae* infestation, additional quantitative stereological analyses were performed, aiming to objectively quantify the morphological renal changes and connect them to PKD pathogenesis. The median kidney volume of diseased brown trout was significantly higher, as compared to the baseline values of healthy brown trout from the Wielenbach fish hatchery ($p = 0.0238$, Mann–Whitney test; Figure 9A). There was a significant increase of the relative and absolute volumes of interstitial renal tissue ($p = 0.0238$, Mann–Whitney test; Figure 9C).

The median relative volume of non-interstitial kidney tissue (kidney functional units [KFU]) consisting of glomerular, tubular, and vascular compartments was significantly decreased in diseased brown trout ($p = 0.0238$, Mann–Whitney test). However, the absolute KFU volume was not significantly decreased ($p > 0.05$, Mann–Whitney test; Figure 9D). The increase of total kidney volume in diseased brown trout was thus mainly due to a significant increase in interstitial tissue. Furthermore, the absolute volumes of melanomacrophage centers in the kidney of diseased and healthy brown trout were not significantly different ($p > 0.05$, Mann–Whitney test; Figure 9E). In healthy brown trout, no parasitic pseudoplasmodia could be detected. In diseased fish, parasitic pseudoplasmodia occupied only a minimal amount of kidney volume (Figure 9B), and no statistically significant differences between the absolute volumes of tubular and interstitial parasitic stages were detectable ($p > 0.05$, Mann–Whitney test).

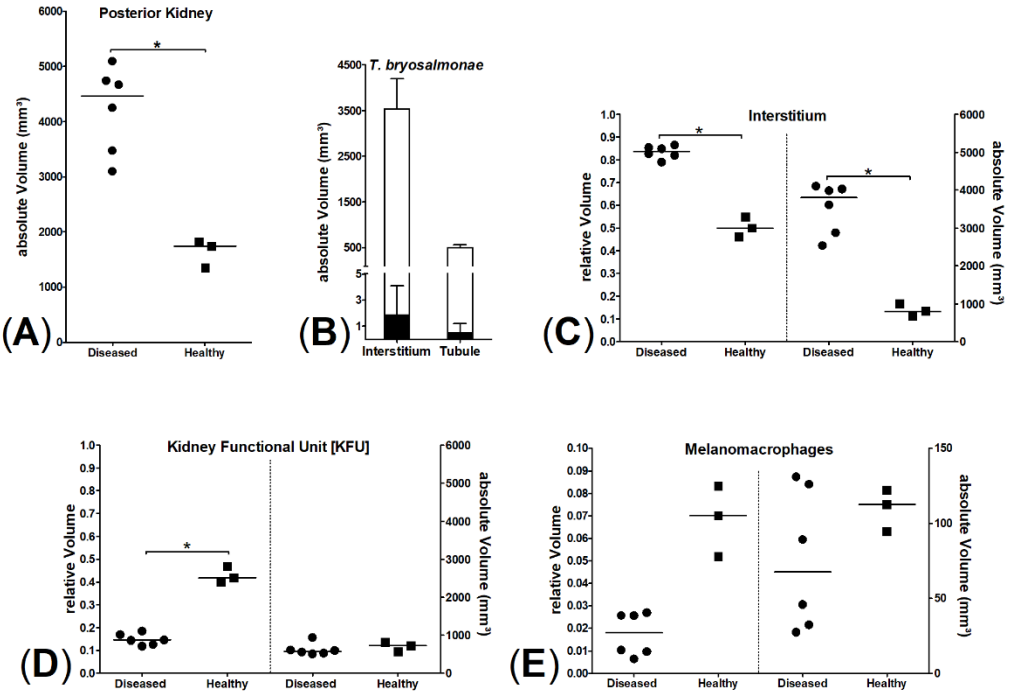

Figure 9. Stereology of posterior kidney of six diseased and three healthy brown trout; significant differences marked by * (p = 0.0238, Mann–Whitney test). (**A**) Comparison of posterior kidney volumes, (**B**) parasite volumes (interstitial and tubular parasite stages in sum, black bars) compared to mean interstital and tubular volumes (white bars), (**C**) interstitium, relative (left) and absolute (right) volumes of interstitial kidney tissue, (**D**) kidney functional unit [KFU], relative (left) and absolute (right) volumes of KFU, (**E**) melanomacrophages, relative (left) and absolute (right) volumes of melanomacrophages.

Discussion

Due to introduction of rainbow trout into German rivers in the early 19th century, autochthonous brown trout populations had to face a strong habitat and food competitor which led to a brown trout decline or even disappearance in many Southern German rivers (Holm 2014). With the advent of climate change, the infection pressure on fish increases with rising water temperatures (Marcos-Lopez et al. 2010). Two diseases especially devastating for brown trout are influenced by water temperature: PDS and PKD (Schwaiger 2013, Wahli *et al.* 2008). Over the last decades, local anglers have reported a massive decline of the brown trout populations in the grayling zone of several pre-alpine rivers in southern Germany. These mass mortalities were mostly attributed to PDS, because the skin of affected fish often turned dark. In the pre-alpine Isar River, similar observations were made by local anglers and therefore investigations to identify a cause for the decline were initiated.

In this field study we investigated the reported mortalities in the Isar River within

the urban area of Munich using state-of-the-art histological, quantitative-stereological, and molecular assays. With these methods we were able to identify and link two major diseases of brown trout, PDS and PKD, to the decline of Isar brown trout. Both conditions can result in rather unspecific skin darkening and are considerably influenced by water temperature (Schwaiger 2013, Clifton-Hadley 1984, Schmidt-Posthaus *et al.* 2012).

PDS leads to high mortalities in affected brown trout in the summer months (Schwaiger 2013). Investigations to identify the cause of PDS have been ongoing for decades, however, up to this date the postulated causative pathogen remains elusive (Fux *et al.* 2019). So far identification of the syndrome relies on histological examination of the affected brown trout. Hepatic necrosis and splenic white pulp depletion with or without gastrointestinal edema are the hallmarks of the disease (Schwaiger 2013). In the present study, we chose clinical and histological features to define the presence of PDS: Brown trout had to show skin darkening and combined hepatic necrosis and splenic lymphocytic depletion. Using this definition, four fish were identified showing the same distribution and quality of lesions when compared with the observations in an established controlled PDS-exposure experiment (Fux *et al.* 2019). We are aware, that in our study only few brown trout met the criteria for PDS. This indicates that the definition might be too strict and we miss out some fish with PDS, which only show few/singular lesions. For example, three additional fish displayed hepatic necrosis without splenic lymphocytic depletion. In the aforementioned controlled PDS exposition trials from 2008 and 2009 the extent and coincidence of hepatic and splenic lesions also displayed considerable variations between individual fish affected by PDS (Schwaiger 2013). Furthermore, identification of histologic lesions requires a certain threshold of damage, thus making histology a diagnostic tool far less sensitive compared with a nucleic acid detection assay like qPCR. It is tempting to speculate that the true number of PDS-affected brown trout might actually be quite higher than those which met the definition criteria.

To this date, a molecular biologic tool to sensitively detect PDS is not available. Although a piscine orthoreovirus (PRV-3) was recently suggested as the causative

agent of PDS (Kuehn *et al.* 2018), a follow-up study could not confirm a direct association between PRV-3 infection and PDS. The qPCR experiments verified viral nucleic acid not only in healthy control brown trout but also failed to detect it in some diseased animals with histologically confirmed PDS (Fux *et al.* 2019). Thus, the potential impact of reovirus infections on the health status of brown trout populations remains unclear. In this study, the PRV-3 specific qPCR on liver samples was negative in all samples.

Like PDS, PKD can also lead to high mortalities, especially in farmed trout (Ferguson 1979). The majority of moribund brown trout in this study were positive for PKD, which is consistent with findings of other studies (Schmidt-Posthaus et al. 2017, Lewisch et al. 2018). PKD spreading in wild brown trout populations has been reported before (Schmidt-Posthaus et al. 2015, Dash and Vasemagi 2014). In total, 94% of the brown trout and 66% of the rainbow trout were qPCR positive for PKD. These results suggest an enzootic distribution of its cause *T. bryosalmonae* within the Isar River. Interestingly, positive rainbow trout showed a similar distribution of ΔCq-values but did not display apparent systemic signs like black skin discoloration or emaciation. The cause of this finding remains speculative; maybe rainbow trout are more robust to environmental changes and can therefore control parasitic infestation.

To accurately estimate the impact of PKD infestation on brown trout health, we implemented quantitative stereological methods in 2018. Clifton-Hadley (1987b) indices determined in 2017 indicated mild to moderate macroscopic lesions of PKD in brown trout posterior kidneys. However, this semiquantitative scoring is subjective and prone to errors in mildly affected individuals, thus we opted to examine the posterior kidney as PKD target organ using design-based quantitative stereology. This technique encompasses a body of sampling and analysis methods based on the principles of stochastic geometry to receive accurate (i.e., precise and unbiased) estimates of quantitative morphological parameters (such as volume, surface area, or particle numbers) of three-dimensional structures by analysis of two-dimensional physical or optical section planes of these structures. This is done without making assumptions on the size, shape, and orientation of the structures of interest (Tschanz et al. 2014, Howard

and Reed 2005). Using appropriate quantitative stereological analysis methods, we were able to demonstrate a highly significant effect of *T. bryosalmonae* infection on the increase of the interstitial tissue compartment of the posterior kidney with unbiased methods when compared to previous studies (Clifton-Hadley 1987b). Even in kidneys with relatively low Clifton-Hadley scores, the volume of interstitial tissue*,* i.e., hematopoietic and inflammatory cells, was significantly increased. In PKD-affected trout the degree of renal damage and inflammation is connected to the manifestation of clinical signs in affected fish (Clifton-Hadley 1987b).

In addition, the volume of melanomacrophage centers (MMCs) in the posterior kidney was decreased in moribund fish, however, not significantly. MMCs are regarded as immunological kidney guardians and are very plastic immunologic structures. Remodeling of MMCs in fish depends on sex, age, and possibly on occurring infections (Agius and Roberts 2003, Steinel and Bolnick 2017). The findings of this study hint that PKD might have a detrimental effect on MMC volume and possibly MMC function. However, for the detection of a significant effect of PKD on MMC volume, the number of fish in our study was too low.

Recently a link between PDS and PKD has been suggested, with PKD inducing PDS likely via immunosuppression (Gorgolione 2016). PKD was also detected in the four brown trout with defined PDS lesions. Although there is some lesion overlap between PKD and PDS, hepatic necrosis is clearly not a distinctive feature of PKD (Hedrick 1993, Clifton-Hadley 1984, Abd-Elfattah *et al.* 2014). To further distinguish PKD from PDS, additional qPCR-tested samples of several brown trout derived from a controlled PDS exposition trial were used to detect PKD infestation (Fux *et al.* 2019). Of these, 2/29 posterior kidney samples from PDS-negative animals were PKD-positive by qPCR. None of the PDS-affected brown trout with combined hepatic and splenic lesions, such as liver necrosis and depletion of the lymphocytic cell population in the spleen, were positive for *T. bryosalmonae* (n = 39, qPCR and histology, data not shown). This finding indicates that PDS can be separated from PKD through characteristic PDS liver and spleen pathology. Furthermore, the two diseases can occur both combined and separately whereby each individual illness can lead to high mortalities within

affected brown trout populations.

During the sampling months, the Isar had constantly increased water temperatures. Variations in water temperatures in river systems are not unusual. Increased water temperatures enhance PKD due to positive effects on the development of the parasite and increased severity of pathological lesions (Bettge *et al.* 2009). Besides, onset of PDS can be delayed by low water temperature, suggesting that the opposite might also be true and higher water temperatures might enhance PDS (Schwaiger 2013). Moreover, a constantly high water temperature likely induces environmental stress for brown trout because they are native to an oxygen rich environment. Long-lasting high water temperatures—as measured during the sampling periods—reduce the oxygen binding capacity and thereby induce stress in affected fish and promote their vulnerability for infectious pathogens (Kittilsen *et al.* 2009, Leclercq 2010). In this context, the black discoloration of brown trout—as more or less an unspecific symptom–could have been further aggravated by environmental stress (Kittilsen *et al.* 2009). Thus, similar PKD parasite loads (*vide supra*) in brown trout and rainbow trout might not lead to similar disease effects.

Fish mortalities can also be induced by ecotoxicological events (Bavarian Agency of Environment, https://www.lfu.bayern.de/analytik_stoffe/fischsterben/festgestellte_ursachen/index.htm, accessed on 04.08.2019). Although we did not measure water parameters directly, we consider such events unlikely as cause for the observed brown trout mortalities. First, during the last several years, only brown trout were repeatedly observed to be affected over a period of at least two months, which makes a single ecotoxicological event unlikely. Second, no other fish species were affected. Finally, water parameters are regularly analyzed by the Bavarian Agency of Environment which did not communicate ecotoxicological events during the sampling periods. To exclude bacterial causes for the brown trout mortalities we performed bacteriologic culture of several organs. *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in trout, was not detectable and *Aeromonas salmonicida* could only be cultured from one fish which did not display any lesions of furunculosis. Since histology

also failed to reveal lesions caused by bacteria or bacterial colonies, we conclude that the primary cause of the mortalities likely was not a bacterial infection.

In conclusion, PKD and PDS are both linked with the brown trout decline in the pre-alpine Isar River. Characteristic lesions of PKD were observed in several fish and parasite infestation was readily detectable with all applied methods (immunohistochemistry, qPCR, and stereology) and connected to considerable posterior renal alterations. Immunological compromise induced by PKD in combination with environmental stress derived from high water temperature appears likely to further aggravate the vulnerability of brown trout for other infections. As shown within this study, PDS also affects the pre-alpine Isar River. Diagnostics on PDS solely based on histological examinations identified only few brown trout being affected by PDS. As PDS lesions vary and histology is not a very sensitive tool, the real number of PDS-affected brown trout in this study might be considerably higher. Further studies on PDS are needed to identify its causative agent by biomolecular examinations to initiate the development of countermeasures for preservation of autochthonous brown trout populations in pre-alpine rivers. Moreover, the identification of the cause of PDS will enable future experiments to investigate the independence or the link of PDS and PKD.

Materials and Methods

Sampling Sites in the Isar River and Fish Specimen Collection

The fish used in this study originated from the Munich city segment of the 292 kilometer-long Isar River including the meadows. This Isar River section underwent exceptional re-naturalization in major efforts to improve and protect the river environment during the last decades. While recognizing the pressures of an urban environment within the sampling area, the removal of concrete or inert constructions in the riverbed and on riverbanks and the replacement with vegetation structures alleviates major damages and allows to restore biodiversity. Typical fish species are known to have functional reproduction and hiding places in this renaturalized habitat and include i.e., Danube salmon (*Hucho hucho*), common barbel (*Barbus barbus*), grayling (*Thymallus thymallus*), and common nase (*Chondrostoma nasus*). The “Die Isarfischer München e.V.”, the

tenant of the water body “Isar city”, gave permission for river access in this conservation area. This study was conducted in accordance with national and federal guidelines for animal welfare (German Animal Welfare Act, Tierschutzgesetz; Bavarian Fishery Act, Bayerisches Fischereigesetz). Exclusively adult moribund brown trout were caught and humanely euthanized. No ethical approval was required.

A distance of twelve river kilometers (48°04'28.7"N 11°32'27.5"E-48°07'41.1"N 11°34'49.7"E) was inspected daily by foot along the river banks to identify and collect adult moribund brown trout, starting at the beginning of August until the end of September in 2017 and 2018. This section of the Isar River was chosen, because dead/moribund brown trout were reported by anglers from this area and accessibility was given. In this river segment, the flat shore zones consisted of mainly gravel substrate. Moribund fish were observed in the main stream (in flat shore zones without strong water current) and in the meadows (Figure 1). Before collection, swimming behavior (velocity, positioning, and flight reflex) and general condition (activity, body color, body condition) were assessed. The fish were caught with a modified fish basket made of thin wire. The basket was round (60 cm in diameter) and both ends were open. Adult moribund brown trout were slowly surrounded with the basket to avoid additionally stress. The basket was pushed down to the ground and fish were carefully caught with a landing net. Fish were euthanized with Tricaine methane sulphonate (1 g/10 L).

After the 2017 sampling period and the initial identification of PKD infection of brown trout, we additionally monitored the Isar River trout population for PKD within the same river segment. Due to local regulations, only fly and spin fishing were allowed to catch trout. Selected fly anglers and two of the authors (DA and GS) caught trout in 2018 by fly fishing. Every angler submitted samples of posterior kidneys from caught trout during May until October 2018. All fish were actively feeding on the fly lure. Anglers reported that the fish showed no obvious signs of organic disease, their nutritional status was good, and their stomachs were well filled. To avoid interobserver variability, trout posterior kidneys were not scored using the in Clifton-Hadley index. However, only rainbow trout were caught during this sampling activity. In these, pathological examination was

carried out only infrequently. In total, 50 rainbow trout posterior kidney samples were collected, deep frozen and stored for subsequent examination by qPCR.

All efforts to sample healthy brown trout as control fish from the Isar River failed in 2018 and even discovering and sampling of moribund fish was quite challenging. For baseline values of quantitative stereological kidney analyses, we therefore used three healthy brown trout of the same size as the affected brown trout. These fish were raised and maintained in spring water in the hatchery of the Bavarian Agency of Environment (Wielenbach).

Necropsy, Organ Sampling, and Examination

Every brown trout was necropsied directly after euthanasia and macroscopically examined. A comprehensive organ set sample (brain, gastrointestinal tract, gills, gonads, anterior and posterior kidney, heart, liver, and spleen) for histology was routinely fixed 24–48 h in 4% formaldehyde solution, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE).

The same organ set was sampled from every brown trout for biomolecular assays (deep frozen −80 °C) from all individuals.

Bacterial cultures were grown from heart, liver, spleen, and kidney samples using blood and Gassner agar plates. Grown bacterial isolates were picked from agars and specified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy in a Bruker Daltonik MALDI Biotyper. To exclude infection with *Renibacterium salmoninarum*, two brown trout caught in August 2017 in the same river segment were additionally submitted to the Bavarian Health and Food Safety Authority (Erlangen).

Immunohistochemistry

Tissue sections of anterior and posterior kidneys, liver, and spleen of all 31 brown trout underwent immunohistochemistry to detect *T. bryosalmonae* antigens. Heat-induced epitope retrieval (microwave pressure cooker for 33 min) with a tris buffer at pH 9.0 was used for antigen demasking, followed by avidin biotin blocking and normal goat serum to block unspecific reactions. After determination of the final dilution, a monoclonal mouse antibody (IgG1, P01,

Aquatic Diagnostics Ltd, Scotland) was used as the primary antibody (1:50 in tris-buffered saline). To increase sensitivity a biotinylated goat anti-mouse antibody served as the secondary antibody (Vector, BA-9200, Burlingame, CA, USA). After incubation with avidin-coupled alkaline phosphatase (ABC-AP, Vector, AK-5000, Burlingame, CA, USA), Liquid Permanent Red (K0640, Dako Agilent, Glostrup, Denmark) was used as the chromogen. Positive (PKD-infected fish) and negative (substitution of the primary antibody with an irrelevant mouse IgG) controls were included in each assay.

Molecular Biological Investigations

DNA and RNA were isolated from posterior kidney samples using the QIAamp Mini Kit or QIAamp RNeasy Mini Kit, respectively, (Qiagen, Hilden, Germany) according to manufacturer's instructions. For real time PCR the QuantiTect probe PCR kit or the QuantiTect probe RT-PCR kit were used, respectively. Conventional PCR was performed using the Ready Mix Taq PCR Reaction Mix (Sigma Aldrich, Merck, Darmstadt, Germany).

A TaqMan assay for qPCR was used to detect the 18s RNA gene of *T. bryosalmonae* in renal tissue samples. Oligonucleotide primers (F: 5'-TGT CGA TTG GAC ACT GCA TG; R: 5'-ACG TCC GCA AAC TTA CAG CT; 800 nM each primer by Grabner and El-Matbouli (2009) were combined with a newly designed TaqMan probe (5'-FAM-TGG ACA AAC GCA AGC TCC TGA TCT -BHQ1; 400 nM). The thermal profile of the PCR was 95 °C for 15 min, and 42 cycles of 95 °C for 15 s, and 60 °C for 1 min. To verify the correct detection of the *T. bryosalmonae* 18S gene, a highly conserved 435 bp fragment was amplified and sequenced using the primers (F: 5'-CCT ATT CAA TTG AGT AGG AGA; R: 5'-GGA CCT TAC TCG TTT CCG ACC; 500 nM each primer) described by Kent (1998). The amount of parasite DNA is shown with ΔCq-values, meaning ΔCq-values = 42-Cq. For phylogenetic analysis of T. *bryosalmonae*, we amplified (F: 5'-GAA TGA CTT AGC GAG AAC TTG GTG GTA; R: 5' CGC AGC AAG CTG CGT TCT TCA TCG A; 500 nM each primer) and sequenced a 585 bp genome fragment extending from the 18S through the internal transcribed spacer 1 (ITS-1) and terminating in the 5.8S (Henderson and Okamura 2004). The PCR products were controlled by agarose gel electrophoresis and sequenced using the PCR primers and the sequencing service

of Eurofins Genomics (Ebersberg, Germany). For the alignment and analysis of the obtained sequences, DNASTAR Lasergene and MEGA7 software were used. A clustalW algorithm was used for alignment, and a phylogenetic tree was constructed using the maximum likelihood method (HKY matrix) according to Henderson and Okamura (2004).

To exclude an infection with the piscine orthoreovirus 3 (PRV-3), we used a RT-qPCR (Fux *et al.* 2019), targeting the S1 segment of the virus in liver samples (F: 5'-ATC TCT GGC ACC ACA AGA TTT; R: 5'-GAC CAT AGC AGG CTT AGC RTT A; 800 nM each primer; probe 5'-FAM-AGA CAG ACC AAY CCK ATG CCC GC-BHQ1; 300nM). To denature viral dsRNA, the eluate was incubated for 10 min at 95 °C before RT-PCR. The thermal profile of the PCR was 50 °C for 30 min, 95 °C for 15 min; and 42 cycles of 95 °C for 15 s, 57 °C for 20 s, and 68 °C for 40 s.

Quantitative Stereological Analyses

The volumes of distinct tissue compartments (i.e., the volumes of functional kidney unit (KFU), of interstitial tissue, of melanomacrophages, and of parasitic pseudoplasmodia) within the posterior kidney were analyzed in six diseased brown trout and three healthy brown trout (raised in spring water), using unbiased quantitative stereological analysis methods. From each fish, five thin transversal slabs (of ~5 mm) of fresh kidney tissue were sampled for PCR investigation, using a systematic uniform random (SUR) sampling design (Howard and Reed 2005). Subsequently, the kidneys were fixed in situ (whole fish carcasses with removal of all inner organs except the kidneys) by immersion in neutrally buffered 4% formaldehyde solution for 24–48 h. After fixation, the kidneys were carefully excised from the fish carcasses, completely sectioned into equidistant (~5 mm thick), parallel, transversal tissue slabs (Tschanz *et al.* 2014). The total kidney volumes (V_{kid}) were determined from the section areas of the tissue slabs, using the Cavalieri principle, as described earlier in detail (Howard and Reed 2005, Blutke and Wanke 2018). One third of the tissue slabs of each kidney (7–9 slabs/fish) were SUR sampled and embedded in paraffin, maintaining the orientation of their section surfaces. The relative volumes of the different tissue compartments within the kidney ($V_{V(tissue/kid)}$) were determined from the fractional areas of their section profiles and the area of the total renal tissue in

HE stained histological sections. The section areas of distinct tissue compartments were determined by point counting (Howard and Reed 2005) in 12–14 SUR selected fields of view (FOV) per slab at 400x magnification. Per case, 10747 ± 1183 points were counted. The total volumes of different tissue compartments ($V_{(tissue,kid)}$) were calculated from their respective volume fractions within the kidney and the total kidney volume ($V_{(tissue,kid)} = V_{V(tissue/kid)} \times V_{kid}$).

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V. DISCUSSION

Mortalities of brown trout populations in the pre-alpine Isar river have been recognized over the last years by the Bavarian Association of Fishery (“Landesfischereiverband Bayern e.V.") and the "Die Isarfischer e.V.” directly in the city center of Munich. Fish were found moribund or already dead within the late summer months mainly within a specific river segment with good accessibility through flat shore zones. Especially adult brown trout, capable of reproduction, were affected. Other fish species were not affected excluding a general event of fish mortalities. Fish displayed black discoloring of the skin and apathetic behavior, matching the leading symptoms of Proliferative Darkening Syndrome of brown trout known from several pre-alpine rivers in Bavaria. However, no further investigations of these observed mortalities had been done to include or exclude participation of PDS or even participation of other commonly known fish pathogens. The objective of this current study was to provide a general insight in ongoing brown trout mortalities in the pre-alpine Isar river to enable a base for future protection and stabilization programs of brown trout populations in order to sustain an autochthonous population.

Participation of Proliferative Kidney Disease and Proliferative Darkening Syndrome in Isar brown trout mortalities

The emerging disease PKD is known from a lot of European countries (Gorgolione 2016, Vasemagi et al. 2017, Wahli *et al.* 2008, Okamura 2011, Seagrave 1981), but has not yet been described for the Isar river. Interestingly, most studies were done on rainbow trout and the quite complex relationship with the invertebrate host *Fredericella sultana* (Abd-Elfattah et al. 2017, Palikova et al. 2017). Mortalities of farmed rainbow and brown trout were the initial impact of PKD infection (Clifton-Hadley 1984). Especially losses of the young-of-the-year fish in huge farms and the considerable economic aspect probably lead to the research activities starting more than three decades before (Ferguson 1979). Infection of wild river systems seems to be inevitably reliant on the appearance of bryozoan colonies, however other aspects could interrupt this assumption: *T. bryosalmonae* can be originated from bryozoans, infected salmonids or parasitic fragments within the river water (Fontes et al. 2017). Dispersal within infected water bodies and even between infected and non-infected water bodies is possible. Both bryozoan and fish hosts act as a carrier (Okamura 2011). Nevertheless, waterfowl-mediated transport or human-mediated transport of *T. bryosalmonae* infected material within or between water bodies leads to further distribution of the parasite (Okamura 2011, Figuerola *et al.* 2004). Prevalence and spread of *T. bryosalmonae* had been evaluated in Switzerland (Wahli et al. 2007, Wahli et al. 2002). PKD had been found in salmonid species (rainbow trout, brown trout and grayling), whereas mostly farmed rainbow trout and brown trout from rivers were affected. Prevalence of PKD-infection at different sampling sites with >10% was found, indicating that the presence of *T. bryosalmonae* in a river system and infection are commonly correlated (Wahli *et al.* 2007). On the other hand, the presence of PKD varies between regions (Rubin et al. 2019). Recently, PKD was linked to act as a contributing factor for observed brown trout decline in Austria (Gorgolione 2016).

In contrast to Switzerland or Austria, data about the presence or absence of PKD is missing in river systems of Bavaria. Beside lacking knowledge about fish pathogens like PKD in Bavaria, an additional, quite elusive phenomenon is responsible for the decline of brown trout populations: Proliferative Darkening

Syndrome (Schwaiger 2013, Hanfland *et al.* 2013). Participation of Proliferative Darkening Syndrome (PDS) of brown trout, known from a lot of pre-alpine rivers in Bavaria was suspected as a possible reason for observed Isar brown trout decline matching the macroscopically clinical signs described for PDS, such as darkening of the skin and extensive mortalities (Schwaiger 2013).

In our study at the pre-alpine Isar river, 31 brown trout, sampled during warmer summer months (Auguste until September) displayed symptoms like black discoloration of the skin and apathetic behavior. Moribund brown trout displayed mainly internal posterior kidney lesions. Infection with the parasite *T. bryosalmonae* was confirmed by histology, immunohistochemistry and biomolecular assays (Table 1). Pathological examination displayed lesions consistent with PDS (combined lesions of liver necrosis and depletion of the white splenic pulp) in a few moribund brown trout of both years (*n*=4, Table 1).

Table 1.: Distribution of pathological lesions of liver and spleen found in moribund Isar brown trout (*n*=31)

Etiology	**Target Organ**	**Method**	**Result**
PKD	Liver	PCR IHC	29/31 + 14/31 +
PDS	Liver Spleen	Histology Histology	7/31 (Hepatic necrosis) 29/31(Depletion of white pulp) Combined splenic and hepatic lesions: 4/31

Both, PDS and PKD, result in the rather unspecific symptom of black coloring of the skin and are influenced by water temperature (Schwaiger 2013, Clifton-Hadley 1984, Schmidt-Posthaus *et al.* 2012). Clinical signs of affected Isar brown trout were obviously the dark color of the skin and apathetic behavior. Low or even total loss of swimming velocity, noticeable by their avoidance of the drift, proved a highly reduced general condition of affected Isar brown trout.

Like humans, fish have their individual skin color (Sterba 2002). Salmonids are able to produce a black pigment called eumelanin. Further pigmentation of salmonid skin can be stress-induced (Kittilsen *et al.* 2009). Khan and colleagues (2016) recommended a new model for stress response-associated pigmentation. Missense melanin stimulating receptors can lead to low or high stress tolerance within rainbow trout. Consequently, two models following different patterns within the molecular pathway response were proposed based on the release of adrenocorticotropin. Additional, variation of pigmentation (small or large spots) can occur (Khan *et al.* 2016). Black coloring is known as leading symptom for infections with viral (e.g. Viral Hemorrhagic Septicemia (VHS) or Infectious Hematopoietic Necrosis (IPN)), parasitic (e.g. PKD) or unclassified fish pathogens (Amlacher 1981, Schwaiger 2013). Therefore, progressed pigmentation of salmonid skin is a rather unspecific symptom (Leclercq 2010), which does not directly provide a link on the specific, ongoing infection.

According to the suggested stress-based model of Khan (2016), environmental stressors are probably one big, underestimated group beside infectious pathogens. Due to good accessibility directly in the center of Munich, passenger volumes and associated leisure activities, such as stand-up paddling or boat activities, increased at the Isar river (Schnell 2019). But stressors do not only exist directly in or on a water body, animals and plants beside the water body also get in touch with human activities resulting in littered shores or trampled ecosystems (Schnell 2019).

The hypophysis-hypothalamus axis reacts in response to stress (Armario et al. 2012). Different biotic and abiotic stressors can provoke a molecular answer (Schulte 2014). Studies on goldfish (*Carassius auratus*) demonstrated that stress exposed fish are capable to handle one stressor with metabolic restitution,

whether two or even more stressors lead to massive metabolic imbalances. Both in common is the effect on the general condition of the fish (Gandar et al. 2017).

PKD infection goes ahead with different diseases dynamics in age classes and susceptibility of species. PKD related mortalities within farmed trout normally affect fingerlings or young of the year trout (Clifton-Hadley 1984, Hedrick 1993).

Prevalence as well as sensitivity in susceptible species can vary within a river system (Feist 2002, Schmidt-Posthaus *et al.* 2013). In wild trout populations, prevalence of PKD seems to be not directly age-related (Feist 2002, Dash and Vasemagi 2014). In a study, carried out in wild rivers of Estonia, parasite prevalence in juvenile fish was quite higher in the age class 1+ (one summered fish) than in 0+ (young-of-the-year fish). Additionally, adult returning sea trout spawners were positive for PKD (Dash and Vasemagi 2014). Grayling (*Thymallus thymallus*) seem to have a lower susceptibility for PKD than brown trout (Schmidt-Posthaus *et al.* 2015, Skovgaard and Buchmann 2012). Brown trout, chronically infected with *T. bryosalmonae*, are able to close the life cycle by re-infection of *F. sultana* colonies up to two years post primary infection (Abd-Elfattah *et al.* 2014). Persistence of PKD in brown trout is described at least for five years after exposure (Soliman *et al.* 2018). Most remarkable differences were found in gene expression profiles, susceptible for development of intraluminal sporogonic tubular stages of PKD, between brown trout and rainbow trout both infected with the European strain of *T. bryosalmonae* (Kumar et al. 2015). These findings underline a previously published study, insisting that rainbow trout infected with the European strain of *T. bryosalmonae* are unable to re-infect *F. sultana* colonies (Kumar *et al.* 2013). Resistance to reinfection is possible if trout survived the clinical phase of PKD (Foott and Hedrick 1987, Clifton-Hadley 1984).

Tetracapsuloides bryosalmonae leads to hematopoietic hyperplasia and inflammation in the kidney as well as progressive anemia during the clinical phase (Clifton-Hadley 1987b, Schmidt-Posthaus *et al.* 2013, Clifton-Hadley 1987a). The resulting kidney dysfunction may lead to osmotic disturbances and reduced oxygen binding capacity (Bettge *et al.* 2009, Seagrave 1981). Additionally, immunosuppression due to PKD infection provokes vulnerability to secondary infections such as furunculosis (Holland *et al.* 2003, Seagrave 1981). A combination

of pathological changes directly caused by *T. bryosalmonae* and their resulting impact on fish health and environmental circumstances may enhance mortalities (Seagrave 1981). Also, within the Isar river, mortalities were probably not directly caused by PKD. A role as additional stressor seems more likely.

In contrast, PDS causes mass mortalities of all affected fish. Survival rates within salmonid hybrids are given (Schwaiger 2013). In the pre-alpine Isar river, mortalities occurred only within the brown trout population. Isar brown trout displayed focal necrosis and hemorrhages within the liver, both hallmarks of PDS. As fish do not have defined regions within the liver such as other animals, a pattern of injury (central, midzonal, or periportal regions) of observed necrosis cannot be described. Acute liver failure seems not to be the reason for observed mortalities. A cytokine-mediated injury such as seen in other infectious processes seems likely (Cattley and Cullen 2018). Up to now, the reasonable agent for PDS has not been identified (Fux *et al.* 2019).

Moribund Isar brown trout were mainly over 30 cm body length. Loss of young-of-the-year fish or even smaller one-summered exemplars were probably not noticed, neither in the Isar nor in other rivers. Before being recognized, strong drift or flood may stream affected smaller animals away. Regarding disease dynamics of PKD and PDS, caught moribund brown trout may had their first contact with both diseases. Restock of different fish species is done within the Isar river mainly in order to stabilize the natural population. Twice a year, naive trout with unclear prevalence of PKD from hatcheries were stocked, both in 2017 and 2018.

In an experimental trial with rainbow trout and brown trout, parasite dynamics within the fish host were analyzed (Bailey et al. 2018). Fish were exposed to a low level of *T. bryosalmonae.* Parasitic load was higher in brown trout kept at 15°C than at 12°C. When compared to rainbow trout, kept at 15°C, prevalence was significantly higher in brown trout. Detection of parasitic DNA was firstly possible in brown trout kept at 15°C, whereas in remaining groups detection was not possible until day 15 post exposition (Bailey *et al.* 2018). To give an impression of PKD dynamics within the pre-alpine Isar river, rainbow trout were caught and examined on PKD during sampling period of 2018. These caught rainbow trout additional served as a sort of negative control, as rainbow trout are not known to

get affected by PDS (Schwaiger 2013, Born 2013). Two-thirds of sampled rainbow trout were positive for PKD DNA by qPCR. Results indicate an equal distribution of Δ cq-values over the sampling months. Development of PKD at least in rainbow trout and this study, does not increase in high amount due to higher water temperatures comparable to laboratory studies. In wild rivers no exact dose of spores can be validated (Bailey *et al.* 2018). A similar distribution of Δ cq-values of rainbow trout, even with a lower median when compared to diseased brown trout, was seen. In contrast to this findings, clinical symptoms in brown trout and rainbow trout were quite different, as PKD-infected rainbow trout in this study displayed no clinical symptoms or mortalities. These findings indicate the role of PKD as by-stander infection.

Moribund Isar brown trout were infected with a parasitic strain of *T. bryosalmonae*, which could be assigned into the North American clade (Henderson and Okamura 2004). Rainbow trout are not that adapted to the European strain of *T. bryosalmonae,* causing severe symptoms and high mortalities (Grabner and El-Matbouli 2008). As rainbow trout of this study displayed no symptoms of clinical PKD nor mortalities, it seems unlikely that in one river system, several PKD strains with different virulence affect different fish species. The host response with its individual characteristics due to infection with the parasite reflects the dynamic of PKD (Bailey *et al.* 2018).

Proving that observed mortalities in brown trout were solely based on infection with *T. bryosalmonae* is quite challenging (Skovgaard and Buchmann 2012, Feist 2002). Rainbow trout do have the capacity to fully regenerate from infection (Schmidt-Posthaus *et al.* 2012). Moreover, a first contact with *T. bryosalmonae* is reasonable, as PKD-infected Isar brown trout were adult fish and mortalities due to PKD are mainly seen in young-of-the year trout (Ferguson 1979).

Incubation periods for PDS are quite longer. Fish must be exposed to infectious water for at least four weeks (Schwaiger 2013). Few moribund Isar brown trout displayed clinical symptoms of PDS at the end of Auguste, therefore incubation periods had been fulfilled.

Considering both disease dynamics, it seems likely that naïve, stocked brown trout were confronted with two diseases, which their immune system may had never seen before. Besides, other detrimental factors (e.g. temperature and environmental stress) may had led to a fulminant course resulting in strong immunosuppression and finally death of affected fish.

Detrimental factors influencing the decline of Isar brown trout

As PDS and PKD are temperature-influenced diseases (Schwaiger 2013, Schmidt-Posthaus *et al.* 2012), ecological factors like water temperatures were included in this study.

In this study, temperatures in the Isar river were constantly high during both sampling periods. Temperature rates are measured by the Bavarian Agency of Environment in defined measuring points during the whole year (data accessible on www.gkd.bayern.de). There was no conspicuous difference seen by comparing temperatures levels between both sampling periods though chronological sequence as well as with temperature ranges of preceding years. June and July of 2017 reached maximum temperatures above >19°C (max. 21°C), whereas August was relatively moderate with < 19°C. An increased temperature was seen in 2018. Beginning in May, temperatures were slowly rising, with peak temperatures over 21°C in August 2018. In consequence, higher water temperatures lead to reduced oxygen-binding capacity, an additional stressor for fish.

Climate change provokes inevitable changes within the environment, especially within water systems. Species must adapt on warmer environment. Some species are more capable to compensate the fast-increasing temperature and environmental changes than other species. Even between different size classes of one species, temperature tolerance varies, such as larger brown trout individuals compensate temperature variations better than smaller ones (Elliot and Elliot 2010). According to Comte and Grenouillet (2013), most of stream fish have not shifted towards a higher temperature to face the advent of climate change in their natural habitat. With the upwards-shift in their natural habitat, temperature driven diseases such as PKD will further spread within brown trout populations and resulting in increased infection pressure (Hari 2006, Marcos-Lopez *et al.* 2010, Rubin *et al.* 2019). PDS onset can be delayed by constantly lower water temperatures (Schwaiger 2013).

Sampled Isar brown trout were mainly found in specific river sections. Characteristically for these sampling places were reduced drift and flat shores. Over the last decades, the Isar river underwent re-naturalization especially in the

sampling river sections. With enormous efforts, deadwood and stones were brought in to recreate a natural environment and provide spawning grounds and places of retreat for inhabitants. Fish lifts were installed beside weirs to allow consistency in order to connect biological ecosystems within the Isar river. For some species, such as Danube Salmon (*Hucho hucho*), these efforts resulted in success and reproduction is seen (personal communication, "Die Isarfischer e.V.). Brown trout seem not to be successful in the run of the survival of the fittest as they are more vulnerable for ecological and biological stressors, e.g. temperature (Sterba 2002, Hari 2006, Elliot and Elliot 2010), habitat or food competition (Scott and Irvien 2000, Seiler and Keeley 2009).

Rainbow trout, originally based in the west north of America, were introduced to Europe in the end of the 19th century (Sterba 2002, Crawford 2008). Between the end of the 19th century and the early years of the 20th century, eighteen imports of rainbow trout eggs from the USA to Germany (similar number with imports to France and UK) are documented (Stankovi *et al.* 2015). The risks involved in the import of these newcomers were not considered. Potential or already self-sustaining populations of rainbow trout across Europe are described (Stankovi *et al.* 2015). In comparison to brown trout, rainbow trout are not that demanding in farming, water quality and environmental conditions (Sterba 2002). Consequently, this species has been seen as easy to breed fish and as additional source of food (Halverson 2008). Over the last decades, rainbow trout were stocked by anglers' associations without any control of law (Stankovi *et al.* 2015). Missing control in stocking and the associated impacts on ecological niches may have created a base for nowadays difficulties. Nevertheless, as rainbow trout, the non-native salmonid species, can act as carrier of pathogens from hatcheries in aquatic systems, this potential negative impact on native salmonid species should be considered (Wahli *et al.* 2002, Stankovi *et al.* 2015). The same is also valid for every fish species.

Discussion about displacement of brown trout by rainbow trout are still current and there are a lot of various approaches in Europe and even between federal states of Germany. According to the European Neobiota Order Nr. 1143/2014, *Oncorhynchus mykiss*, is seen as invasive species. In Bavaria, the law of fishery does not prohibit the stock of rainbow trout (Bayerisches Fischereigesetz, BayFIG,

BayRS 793-1-L, (Art. 1–80)). Due to the water framework directive (2000/60/EG), electro fishing is done to examine the appearance of fish species in aquatic river system. According to the latest fish report about occurrence of fish species in Bavarian rivers, rainbow trout are well established (Schubert et al. 2018). According to Stankovi *et al.* (2015) self-sustaining populations of rainbow trout in Bavaria exist in few high-altitude streams in upper Bavaria. Early warning stages for brown trout, the native species in Bavarian rivers, is seen as justified (Schubert *et al.* 2018). Restock of brown trout was done, related with the hope of reproduction and stabilization of the natural population but this practice may conflict with animal welfare (Hanfland *et al.* 2013), as stocking of brown trout in PDS-affected river sections involuntary leads to death of these individuals.

Decline of this native species may be caused by a mixture of reduced reproduction, even with restock, environmental changes like competing species, higher temperatures as well as infectious pathogens.

Future perspectives on stabilization of brown trout populations in Bavaria according to the results of this study done at the pre-alpine Isar river

As consequence of restock, done with fish from various hatcheries, uncontrolled introduction of new or already known fish pathogens might bring us todays or even future problems (Feist 2002, Okamura 2011). *T. bryosalmonae* is a fish pathogen, which can conquer new habitats quite unnoticed. Especially for PKD, infections are often overseen in wild trout populations (Vasemagi *et al.* 2017). Susceptibility of species goes ahead with a wide range of sensitivity. Environmental substances or contributing factors may complicate PKD infection in fish or led to more infectious pressure and increasing prevalence (Feist 2002, Sudhagar *et al.* 2019). German strains of *T. bryosalmonae* are known (GenBank AJ640016–AJ640018) from farmed rainbow trout in Bavaria (Henderson and Okamura 2004). With this study, incidence of PKD spread in the pre-alpine Isar river is verified. As examined trout displayed a high qPCR-prevalence (brown trout 29/31; rainbow trout 33/50) restock with naive fish in Isar water may lead indispensable to infection with the parasite. Sampling sites, once found to be positive for PKD, remain positive and fish get commonly diseased (Wahli *et al.* 2007). Further studies should be done to determine prevalence of PKD in wild river systems of Bavaria. Restock should meet the requirements of specific conditions in individual river systems. Risk involved in restock with especially brown trout, already infected or chronically infected with PKD, would-be long-lasting release of infectious spore material (Abd-Elfattah *et al.* 2014). The same is also true for PDS – in rivers affected by PDS, restock with brown trout from hatcheries will fail as PDS always ends fatal. Critical points, such as animal welfare, should be kept in mind as mass mortalities due to PDS get in conflict with them (Hanfland *et al.* 2013). Infectious diseases, such as listed in the regulation of fish diseases (Fischseuchenverordnung, BGBl. I S. 2315, zur Durchführung der Richtlinie 2006/88/EG), mainly affect hatcheries. There is no regulation set for restock with farmed fish from different genetical background, origin or health status.

Beside infectious diseases, environmental changes, like global warming are an increased risk for sensitive aquatic ecosystems (Hari 2006). A normal natural stabilization of fish populations depends on the possibility of fish passing and

available spawning grounds (Pander et al. 2011, Pander and Geist 2016). Rising water temperatures in combination with hypoxia lead to stress and vulnerability of fish (Anttila et al. 2015). Conditions for external and internal parasites as well as bacterial infections are often linked with higher water temperatures – like in this study Proliferative Kidney Disease and Proliferative Darkening Syndrome.

This field study gave insights in disease dynamics within the pre-alpine Isar river. Missing information and unknown pathogen status were considered before this study was carried out. Differential diagnoses were included.

Work in future must be done to not only stabilize fish populations by restock with the hope of self-preservation, but rather with the focus on fish health to prevent or delay fish diseases. Especially restock of farmed fish, may encourage the risk of carry-over of infectious diseases and the aquatic ecosystems may only tolerate a little amount of changes until final breakdown. We should keep in mind that human mistakes in the past may cost us treasured natural assets in the future – based on the quotation of Albert Schweitzer: *"We live in a dangerous era. The human dominates nature, prior to he has learned to dominate himself*.".

VI.SUMMARY

A decline of brown trout (*Salmo trutta fario*) was observed in the pre-alpine river Isar over the last years. The Bavarian Fisheries Association and local recreational anglers reported high mortalities especially within the warmer summer months. Moribund fish displayed distinct black coloring of the skin, reduced swim behavior and apathy. Other species were not affected. Proliferative Darkening Syndrome, known to be responsible for massive decline of autochthonous brown trout populations within a lot of pre-alpine rivers in Bavaria, was suspected as cause for observed mortalities in the Isar river. An infection with *Tetracapsuloides bryosalmonae*, the causative agent of Proliferative Kidney Disease, was also discussed.

During sampling years 2017 and 2018, a daily inspection of 12 km of the water body was performed (August-September). Moribund, black-colored brown trout were caught, and an extensive organ set was examined macroscopically, histologically and with immunohistochemistry and qPCR. An infection with the parasite *T. bryosalmonae* was found in 93.5% of sampled brown trout (*n*=31). The impact of *T. bryosalmonae* on renal tissue remodeling in infected brown trout was examined in the sampling year of 2018 with unbiased quantitative stereological methods. A significant increase of renal tissue volumes, characteristically for PKD, with hematopoietic tissue hyperplasia and interstitial inflammation was found. The parasitic strain, found in infected brown trout, was grouped into the north American clade by phylogenetical analyses. Pathological findings, characteristic for Proliferative Darkening Syndrome, such as hemorrhages and necrosis of the liver combined with splenic lesions, were found in 12.9% examined brown trout. An infection with piscine orthoreovirus 3 was ruled out by qPCR.

This study highlights the participation of both, PKD and PDS, being associated with decline of brown trout in the pre-alpine Isar river.

VII. ZUSAMMENFASSUNG

Über die letzten Jahre wurde ein Rückgang der Bachforellenpopulation (*Salmo trutto fario*) im präalpinen Fluss Isar beobachtet. Der Landesfischereiverband Bayern e.V. und lokale Fischer berichteten von erhöhten Mortalitäten insbesondere während der warmen Sommermonate. Moribunde Fische zeigten neben einer ausgeprägte Schwarzfärbung ein reduziertes Schwimmverhalten und Apathie. Andere Fischarten waren nicht betroffen. Das Bachforellensterben, welches in vielen präalpinen Flüssen Bayerns zu einem erheblichen Rückgang der autochthonen Bachforellenpopulationen führte, wurde als Ursache der Mortalitäten in der Isar angenommen. Eine Infektion mit dem Parasiten *Tetracapsuloides bryosalmonae*, Auslöser der Proliferative Kidney Disease, wurde ebenso in Betracht gezogen.

In den Jahren 2017 und 2018 wurde eine tägliche Inspektion eines Isarsegments von 12 km Länge durchgeführt (August-September). Moribunde, schwarz gefärbte Bachforellen wurden entnommen und ein umfangreiches Organset wurde makroskopisch, histologisch und mittels Immunhistochemie und qPCR untersucht. In 93,5% der beprobten Bachforellen (*n*=31) wurde eine Infektion mit dem fischpathogenen Parasiten *T. bryosalmonae* nachgewiesen. Mittels quantitativer stereologischer Untersuchungen wurde das Ausmaß des Gewebeumbaus der Rumpfniere, verursacht durch *T. bryosalmonae*, in infizierten Bachforellen des Untersuchungsjahres 2018 geschätzt. Die Rumpfniere zeigte einen signifikanten Anstieg des renalen Gewebevolumens, charakteristisch für PKD, sowie eine Hyperplasie des hämatopoetischen Gewebes und eine interstitielle Nephritis. Mittels phylogenetischer Analysen wurde der gefundene Parasitenstamm der Nordamerikanischen Gruppe zugeordnet.

Pathologische Befunde, charakteristisch für das prä-alpine Bachforellensterben Bayerns, wie Leberblutungen und -nekrosen kombiniert mit Läsionen der Milz, wurden in 12,9% der untersuchten Bachforellen beider Jahrgänge festgestellt. Eine Infektion mit dem Piscinen Orthorevirus 3 konnte mit molekularbiologischen Methoden (qPCR) ausgeschlossen werden.

Diese Studie beschreibt die Beteiligung von PKD als auch PDS am Rückgang der Bachforellenpopulation im prä-alpinen Fluss Isar.

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