On the potential role of *Mergus merganser* as transport hosts for *Tetracapsuloides bryosalmonae*

Running title: Role of goosander for dispersion of PKD

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Abstract

Transmission paths in the distribution of Proliferative Kidney Disease (PKD) of salmonids are still largely unknown. In this study, the role of goosander (*Mergus merganser*) as possible transport host for *Tetracapsuloides bryosalmonae* through faeces was examined. Goosander fledglings were fed exclusively with diseased brown trout (*Salmo trutta fario*). In all trout used for feeding, intra-tubular sporogonic stage of the parasite were confirmed histologically. Between one to ten hours post-feeding, the goosander faeces were sampled and tested for *T. bryosalmonae* DNA. In qPCR, only DNA fragments were found, in conventional PCR no amplification was confirmed. Therefore, we hypothesize that the role of goosander as transport hosts for *T. bryosalmonae* via their faeces can be neglected.

Keywords

Mergus merganser, *Tetracapsuloides bryosalmonae*, brown trout, transport host, faeces

1. INTRODUCTION

Pathogen transport and transmission is a driving force in the dynamics of infectious diseases (McCallum 2001) . In aquatic environments, pathogens are often transmitted indirectly after the pathogen has travelled some distance in the water before encountering their target host (Murray 2009). However, this transmission is often restricted by the limited connectivity of waterways. Water birds play an important role in transporting pathogens over long distances (Hubálek 2004; Jourdain et al., 2007) to new, possibly formerly pathogen free areas. Proliferative Kidney Disease (PKD) is emerging during the last decades and spreading over distant areas in Europe, as Estonia and Iceland (Dash and Vasemägi, 2014; Kristmundsson et al., 2010) and North America (Smith et al., 1984). PKD has contributed to the long-term decline of brown trout (*Salmo trutta*) populations in Switzerland (Okamura et al., 2011; Wahli et al., 2002), Austria (Waldner et al., 2019) and Germany (Arndt et al., 2019), led to massive economic losses in the rainbow trout (*Oncorhynchus mykiss*) production (Feist 2004), and was recently identified as one of the factors in a mass mortality event of whitefish in North America (Hutchins et al. 2018).

PKD of salmonids is caused by the myxozoan parasite, *Tetracapsuloides bryosalmonae* (Myxozoa, Malacospora, Cnidaria) (Canning et al., 2000; Morris and Adams, 2006). Infected bryozoans release spores, that infect fish, mainly young-of-the-year (YOY), by penetrating the gill epithelium and most probably heading towards the vascular lumen (Grabner and El-Matbouli 2010). Through haematogenic dispersion a generalized infection occurs, with the kidney as main target organ (Feist et al., 2001). There the parasite develops into a malacospore, which migrates into the tubular lumen and is eventually excreted via the urine (Hedrick et al., 2004; Strepparava et al., 2018). These malacospores infect susceptible bryozoans (Grabner and El-Matbouli, 2008; Morris and Adams, 2006; Tops et al., 2004). PKD is a temperature dependant disease (Bettge et al. 2009b; Strepparava et al. 2018), asymptomatic at lower temperature, but with an excessive cellular immune response, severe renal lesions and high numbers of malacospores in susceptible hosts triggered by increasing water temperature in late summer / early autumn (Bailey et al., 2017).

The main distribution of goosander (*Mergus merganser*) encompasses Scandinavia, Siberia and North America (https://avibase.bsc-eoc.org). This bird species is also widely distributed in the Swiss midlands, with many breeding populations in Swiss lakes and rivers (www.vogelwarte.ch), including the river Wutach where this study was performed (Fig. 1). During late summer / early autumn, YOY brown trout are the main food source for adult birds and fledglings.

The role of water birds as PKD vectors through feeding on infected brown trout was not examined so far. Therefore, the aim of this study was to clarify whether *M. merganser* acts as a potential transmission vector of PKD by excretion of viable *T. bryosalmonae* malacospores after ingestion of PKD positive YOY brown trout.

2. MATERIAL AND METHODS

2.1 Sampling of brown trout

The river Wutach, at the border between the north-eastern part of Switzerland and southern Germany, was chosen for sampling, based on former investigations showing high infection prevalence in brown trout ranging from 90 to 100% (Schmidt-Posthaus et al. 2015; own investigations). End of August 2019, 46 wild YOY brown trout were sampled at two stretches of 100 m in the river by means of electrofishing (2'677'061.2/1'289'694.7, 2'677'814.2/1'291'059.7). Fish were killed by decapitation, length and weight were measured. An ad hoc complete necropsy was performed and macroscopic changes in the inner organs, especially in the kidney, were evaluated. In ten out of the 46 sampled brown trout showing the most obvious signs of PKD (moderate to severe swelling of the kidney, greyish discoloration, nodular appearance), a small piece of the posterior kidney was immediately fixed in 10 % buffered formalin for histopathological and immunohistochemical examination. The whole fish including all inner organs (except the removed small piece of the posterior kidney) were frozen

84 at -20°C, transported to the Swiss Ornithological Institute, Sempach and fed to the goosanders
85 in the following morning.

86 2.2 Preparation of the goosanders

87 Two juvenile goosanders, approx. 800 g each, kept at the Swiss Ornithological Institute were
88 separated from the group for 24 hours in a 20 m^2 cage with a large wet area. Overnight they
89 were deprived of food for 12 hours and then fed exclusively with the 10 infected whole brown
90 trout, starting at 8 am in the morning. Every hour for the following 10 hours, faeces excreted
91 by both birds were collected as nine separate samples and immediately fixed in RNAlater®
92 (DNA stabilisation solution) for qPCR analysis. The faeces samples were sent to the Centre
93 for Fish and Wildlife Health, University of Bern for further analysis.

94 2.3 Histopathology and immunohistochemistry

95 Formalin-fixed kidney samples were paraffin-embedded and processed for histological
96 examination using routine protocols. Two consecutive sections were prepared. One section
97 of 3 µm thickness was prepared for histopathology (haematoxylin-eosin stain, H&E). H&E
98 stained slides were examined by light microscopy (Nikon Eclipse E400Nikon).
99 Histopathological changes of the whole kidney section were classified from 0 (no alterations)
100 to 6 (severe proliferation of the hematopoietic tissue, multiple areas of haemorrhage,
101 widespread necrosis, multiple thrombi, severe multifocal infiltration) (Bettge et al. 2009b).
102 Additionally, the infection intensity was classified histologically by visual evaluation of the
103 whole slide, ranging from 0 (no parasites) to 6 (high numbers of parasites in renal
104 hematopoietic tissue, vessels and / or tubules). The second slide was used for
105 immunohistochemistry (IHC). For IHC staining of the specific antigen, a monoclonal anti-
106 *Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics Ltd.,
107 Stirling, UK) was used according to a protocol published by (Adams et al., 1992).

108 2.4 DNA extraction and qPCR for detection of *T. bryosalmoae* DNA in goosander faeces

Each faeces sample was homogenized in a 2 ml tube containing 0.4 ml ATL-Buffer (Qiagen, Switzerland) with a 2 mm diameter steel bead (QIAGEN, Switzerland) using a tissue lyser (QIAGEN, Switzerland) with a shaking frequency set at 30 shakes per second for 3 min. Genomic DNA was extracted as previously described (Harun, Wang, & Secombes, 2011). DNA was finally eluted in 30 µl of EB buffer (QIAGEN, Switzerland) and stored at -20°C until qPCR was performed. qPCR was performed targeting *T. bryosalmonae* 18 rDNA (Acc. N.: AF190669) according to (Bettge et al., 2009a). The primer pair PKDtaqf1 (5′–GCGAGATTTGTTGCATTTAAAAAG–3′) and PKDtaqr1 (5′–GCACATGCAGTGTCCAA TCG–3′) and probe PKD (5′–CAAAATTGTGGAACCGTCCGACTACGA–3′) were used. Beside the second sample, where 123 ng DNA was added because of limited DNA amount of the sample, all other reactions were performed with 150 ng DNA. All reactions were carried out in triplicate. Non-target controls (DNAse free water) within the qPCR never showed amplification, while the internal controls (Exo IPC) were always amplified, showing no qPCR inhibition.

2.5 Conventional PCR for confirmation of *T. bryosalmoae* DNA in goosander faeces

PCR was performed according to the protocol by (Morris et al., 2002). Primer pair PKX3F (5'-CTAAGTACATACTTCGGTAGA-3') and PKX4R (5'-CCGTTACAACCTTGTTAGGAA-3') described by (Kent et al., 1998) was used. DNA concentrations used varied between 151 and 860 ng/µl. A positive control sample obtained from kidney of clinically infected brown trout and a negative control of uninfected kidney from fish of a farm with no *T. bryosalmonae* infection were included in the PCR procedure. The products were checked on a 1.5% agarose gel for amplification and molecular weight.

3. RESULTS

3.1 Brown trout PKD infection rate, macroscopy and histology

Ten of the 46 sampled brown trout (22%) showed PKD signs by visual examination, like kidney enlargement, greyish discoloration and multiple nodules of different sizes (Fig. 2a).

Histologically, all 10 animals showed moderate infection rates and renal pathology typical for an acute infection. The pathology was characterized by expansion of the renal interstitium by proliferation of the hematopoietic tissue, infiltration with macrophages, multiple necrosis and hemorrhage. Multiple vessels showed thrombi consisting of erythrocytes, fibrin, inflammatory cells and parasites. In the tubular lumen of all 10 brown trout large numbers of intra-luminal malacospores were visible (Fig. 2b). The presence of *T. bryosalmonae* in all kidney compartments (interstitium, vessels, tubuli) could be confirmed by immunohistochemistry (Fig. 2c). The evidence of intra-luminal stages in the kidney of the YOY brown trout fed to the goosanders is an important precondition for the relevance of the whole experiment.
Data for the remaining 36 sampled brown trout not used in this experiment are shown in supplementary table 1.

3.2 Detection of *T. bryosalmonae* DNA in goosander faeces.

Eight of the nine faeces samples revealed a positive result in qPCR (table 1). Ct values ranged from 33 to 41. The sample Nr.1 (one hour after feeding) showed no result in qPCR, whereas the following ones were all considered positive. However, in conventional PCR no signal was detected.

4. DISCUSSION

The rapid and extensive spreading of PKD during the last decades is due to a complex interaction of multiple factors (Bailey et al., 2018; Bettge et al., 2009b; Dash and Vasemägi, 2014; Gorgoglione et al., 2016; Strepparava et al., 2018; Wahli et al., 2002). The global warming with increasing water temperatures enhancing the growth of bryozoans as well as the development of *T. bryosalmonae* within the non-vertebrate host plays an important role. Eutrophication has a synergistic effect (Okamura et al., 2011). The level of bryozoan environmental DNA (eDNA) can be taken as indicator for the bryozoan biomass and therefore for PKD distribution in a river section (Carraro et al., 2017). Other putative factors,

that can play a role, at least partly, are stocking with infected fish in prior naïve rivers and fish migration. Herbivore fish, like common carp, can excrete hatchable infected bryozoan propagules with the faeces after ingestion (Abd-Elfattah et al., 2017). The role of waterfowl as vector of the disease may also be essential. The transmission of *T. bryosalmonae* from North America to South-West Europe is attributed hypothetically to aberrant water bird migration (Henderson and Okamura 2004). Herbivore birds, like ducks, can also transport passively viable bryozoan propagules via their faeces (Charalambidou et al., 2003; Reynolds and Cumming, 2015). Birds might also carry infected fragments of bryozoans or statoblasts inside their plumage over certain distances within or between water systems, acting as transmission vectors of infected invertebrate hosts (Reynolds and Cumming, 2015).

With the present study we investigated the role of fish eating birds as possible vectors for *T. bryosalmonae* in an experimental model using *Mergus merganser* fledglings. Specifically, we examined their capability to excrete infective *T. bryosalmonae* malacospores via faeces after feeding on PKD positive fish. By qPCR, we found shedding of *T. bryosalmonae* DNA fragments, appearing in the bird excrements two hours after feeding on the infected brown trout and lasting at least until 10 hours post feeding. No amplification products were present in conventional PCR, which could have been further processed for sequencing. We hypothesize that during the intestinal transit, the malacospores were degraded, and no viable spores were excreted. However, even if intact DNA would have been detected in the faeces of the goosanders, infection of naïve bryozoa would be necessary to prove the infectivity of transmitted spores.

T. bryosalmonae belong to *Malacosporeae* (Canning et al., 2000). *Malacosporeae* produce soft-shelled spores (Okamura and Canning, 2003) with limited resistance to external environmental influences. This limited resistance could explain the degradation by proteolytic digestive enzymes during intestinal transit.

Based on the above findings we conclude, that *Mergus merganser* do not contribute to the spreading of PKD by their faeces after feeding on brown trout infected with *T. bryosalmonae,* due to the complete digestion of the parasites infective spores.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

ETHICS STATEMENT

Approval for animal experiments was obtained from the cantonal veterinary Office (Bern, Switzerland) (Authorization LU03/19).

DATA AVAILABILITY

Data are available upon request by the authors

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List of figures

Figure 1: Pair of goosander (*Mergus merganser)* from the river Wutach, Switzerland.

Figure 2: Brown trout (*Salmo trutta*) infected with *T. bryosalmonae*; a. macroscopic signs of PKD, kidney enlargement (white arrow), with greyish discoloration, splenomegaly (white star); b. histological picture of the kidney, intratubular malacospores, (closed arrowheads), c. immunohistological picture confirming intratubular malacospores (open arrowheads) and extrasporogonic stages in the interstitial tissue (arrows with closed arrowheads). HE stain. Bar (b,c) = 25 µm.

Table 1: Results of qPCR investigations of nine faeces samples targeting *T. bryosalmonae* 18 rDNA (Acc. N.: AF190669), Ct values of three runs (triplicates), mean values and standard deviation, numbering of faeces samples are according to the timely excretion

Sample	1	2	3	4	5	6	7	8	9
Ct value run 1	no detectable DNA	33.921	36.421	34.761	35.998	40.096	41.282	40.867	39.836
Ct value run 2	no detectable DNA	33.396	35.456	34.052	35.402	39.78	40.582	41.825	40.051
Ct value run 3	no detectable DNA	33.443	35.286	33.865	35.342	39.41	40.157	41.43	39.121
Mean	no detectable DNA	33.59	35.72	34.23	35.58	39.76	40.67	41.37	39.67
Standard deviation		0.291	0.612	0.031	0.363	0.343	0.568	0.482	0.487