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Research Article

Geographic distribution of *Tetracapsuloides bryosalmonae* infected fish in Swiss rivers: an update

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Abstract. Proliferative kidney disease (PKD) has been recognized as a potential threat to brown trout (*Salmo trutta*) populations in Switzerland. A study performed in 2000/2001 on 139 sampling sites from 127 rivers in Switzerland revealed a wide distribution of fish infected by *Tetracapsuloides bryosalmonae*, the causative agent of PKD. The present study aimed to complement this dataset by studying a further 115 sample sites from 91 rivers and 4 fish farms. Mainly brown trout were investigated for the presence of *T. bryosalmonae* by a combination of macroscopical, histological and immunohistochemical examination. In approximately 56% of the examined sampling sites, *T. bryosalmonae*-infected fish were found. The prevalence of infected fish at individual sites ranged from 0% to 100%. Infection intensity, judged on the basis of histological and immunohistochemical evaluation for the degree of parasite infection, varied greatly between and within sites. PKD-positive sites were found in all areas of Switzerland. The wide distribution of the disease in Swiss rivers indicates that PKD may be a causative factor for the catch decline of brown trout, which was suggested over recent decades in Switzerland.

Key words. Proliferative kidney disease; Switzerland; brown trout; geographic distribution.

Introduction

Proliferative kidney disease (PKD) has become a potential threat to the culture of salmonids in Europe and in North America (Hedrick et al., 1993). The disease has been demonstrated in farmed salmonids and in wild fish (MacConnell and Peterson, 1992; Feist et al., 2002; Wahli et al., 2002). In farmed fish, PKD is not always fatal itself but is often followed by secondary infections which lead to the death of infected fish (Feist and Bucke, 1993) and can cause up to 100% mortality. No data are available on the impact of the disease on wild fish populations. However, a nation wide project (FISCHNETZ) looking for causes of catch decline of brown trout in Switzerland raised the hypothesis that PKD might contribute to this situation (Fischnetz, 2004; www.fischnetz.ch).

The causative agent of PKD, *Tetracapsuloides bryosalmonae* (Canning et al., 2000), belongs to the phylum Myxozoa. The life cycle of the parasite includes bryozoans as invertebrate hosts. This was shown by exposure of naïve fish in tanks with resident infected bryozoans which resulted in infected fish (Feist et al., 2001). Salmonids and pike have been demonstrated as vertebrate hosts. However, the full cycle of *T. bryosalmonae* is not yet fully understood. Experiments to transmit the parasite from fish to fish failed (Ferguson and Ball, 1979; D'Silva

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et al., 1984) and no transmission from bryozoans to bryozoans has been achieved to date (Tops et al., 2004). Release of spores from bryozoans into the environment has been shown (Morris et al., 2002a; Hedrick et al., 2004). An infection of bryozoans with kidney material from PKD-diseased fish was possibly demonstrated once, but failed in a repeat experiment (Morris et al., 2002b). No preliminary control for contamination of the bryozoans used was performed in the experiment described by Morris et al. (2002b). Tops et al. (2004) could not demonstrate transmission of parasites from fish to bryozoans. These findings lead to the question of whether salmonids are dead end hosts and if other yet unknown hosts are included in the cycle (Tops et al., 2004).

In addition to the knowledge of the life cycle of *T. bryosalmonae*, a further key factor to understand the epidemiology of PKD is the knowledge of the geographic distribution of the disease. Few data are available on the occurrence of the invertebrate host. Okamura et al. (2001) showed that the distribution of *T. bryosalmonae* in bryozoan populations is patchy in space and time, which makes epidemiological studies particularly difficult. However, recent findings have given some evidence for cryptic stages of *T. bryosalmonae* in bryozoans (Okamura and Tops, 2003) indicative for a permanent presence of the parasites in the bryozoans. PKD has been demonstrated in single locations in many countries (Hedrick et al., 1993), but results from monitoring studies covering larger connected areas or countries are rare. Seagrave et al. (1981) and Feist et al. (2002) presented the results of studies looking at the distribution of PKD in rivers of England and Wales.

In Switzerland, a first survey on the distribution of PKD was performed in 2000/2001 (Wahli et al., 2002). The present paper presents the results of a second survey in 2004 which aimed to close some geographic gaps in the 2000/2001 survey and to further elucidate the status of PKD in Switzerland.

Materials and methods

Selection of sampling sites

Cantons and fisheries associations throughout Switzerland were asked to identify rivers and brooks where fish had never been investigated for the presence of PKD. Special attention was requested for rivers from which adult fish are caught for offspring production or where young fish are raised for restocking measures. In addition, sites were chosen with the purpose to complete the geographic survey of 2000/2001 (Wahli et al., 2002). A total of 111 sites from 91 rivers were included in the present study. The sites were located in three completely distinct river systems with different tributaries. In addition, samples from four fish farms were taken.

Table 1. Sample size needed to detect disease or prove freedom from PKD of a sample site at a confidence level of 95%. Assumptions for the calculations: Sensitivity (SE) of combined methods at 80 and 90%; specificity (SP) of combined methods = 99.9%, for population sizes of target fish at a site (N) = 500 or 3,000, and minimal prevalence of *T. bryosalmonae* infection at a site when parasite present (min P_{PKD}) = 5 or 10%.

$minP_{PKD}$	SE/SP	N	
		500	3000
5	80/99.9	68	71
	90/99.9	61	64
10	80/99.9	35	36
	90/99.9	31	32

Sample size and sampling methods

In order to calculate the sample size which whould be large enough to exclude the presence of PKD at a sampling site, the statistical approach "freedom from disease" was applied by using the freeware program "Survey Toolbox" (http://www.ausvet.com.au/content.php?page=res_software). Depending on the PKD-prevalence, a sample size of 31 to 71 fish would be needed to detect diseased fish with a confidence level of 95% (Table 1). This calculation is based on the following assumptions: 80% or 90% sensitivity of the diagnostic methods used (macroscopy, histology, immunohistochemistry); 99.9% specificity for the serial combination of methods; a minimum observable prevalence of the disease in the population of 5 or 10% (Feist et al., 2002 and personal experiences) and a population size of 500 or 3000 fish per sample site (realistic numbers that may be encountered on a sampling stretch of 200 m). However, due to manpower, financial, technical and ethical reasons, it was impossible to collect or process that large sample size. Therefore we decided to set the sample size to 20 fish per site (which was finally obtained at 82 of the 115 sampling sites), knowing that there will be difficulties in detecting PKD in sites of low prevalence. For instance, the confidence levels drop to 57–62% for a minimum expected PKD prevalence of 5% in the population if only 20 fish are examined (Table 2). Therefore, from a population with a disease prevalence of 5% we would detect in 57–62% of the cases one or more diseased fish. In populations with a disease prevalence of 10%, the confidence level is 82–85%. At sites with a higher PKD prevalence, the sample size of 20 fish is sufficient to detect the disease.

The sampling period took place from the beginning of August to the end of October 2004. The target fish were young-of-the-year. Sampling was done by electro fishing, except in four fish farms where fish could be netted. The farmed fish had been raised in earthen ponds at low densities and were produced for restocking purposes. Fish were caught and kept alive until further processing. After euthanasia with an overdose of clove oil (100 ppm),

Table 2. Probability (%) of observing at least one *Tetracapsuloides*-infected fish in a sample of 20 animals from a population with PKD. Assumptions for the calculations: Sensitivity (SE) of combined methods at 80 and 90%; specificity (SP) of combined methods = 99.9%, for population sizes of target fish at a site (N) = 500 or 3,000, and minimal prevalence of *T. bryosalmonae* infection at a site when parasite present (min P_{PKD}) = 5 or 10%.

$minP_{PKD}$	SE/SP	N	
		500	3000
5	80/99.9	57	57
	90/99.9	62	61
10	80/99.9	82	82
	90/99.9	86	85

length and estimated age were recorded (75% were estimated to have the target age of <1 year). Fish were opened ventrally and assessed for macroscopic symptoms of PKD (exophthalmia, darkening, ascites, general anaemia and kidney hyperplasia). Subsequently, kidneys were excised and fixed in 4% buffered formalin.

In total, 2085 fish were analysed whereof 94% were brown trout (*Salmo trutta*). The remaining 6% were composed of rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and grayling (*Thymallus thymallus*). The data presented include all samples irrespective of the species.

Preparation of kidneys for histology and immunohistochemistry

Fixed kidneys were processed for paraffin-embedding according to routine methods. One haematoxilin & eosin (H&E) stained slide containing the full length of the kidney was produced per fish. For immunohistochemical staining, an anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics Ltd., Stirling, Scotland) was used. The staining procedure was based on a protocol by Adams et al. (1992) with minor modifications. Briefly, unstained sections were incubated overnight using an antibody dilution of 1/100. Unspecific background staining was blocked with goat serum. A biotin-streptavidin-horseradish peroxidase staining kit (Kit Dako LSAB 2 System HRP Code Nr. K0675; DakoCytomation, Zug, Switzerland) followed by AEC (Amino-Ethyl-Carbazole) staining (DAKO AEC K3464) was used to visualize antibody-antigen complexes. Counterstaining of the sections was not performed. Positive control sections were taken from fish with known positive infection status, negative controls from uninfected kidneys were derived from farmed fish with no history of *T. bryosalmonae* infection.

Assessment of samples for PKD

Three different methods were performed: macroscopic examination, histology and immunohistochemistry. A serial combination of these methods was considered the most reliable means to detect infected fish and to reflect the true infection status. The assessment using the various methods was performed as follows:

a) Fish from 114/115 sampling sites in rivers and farms were assessed macroscopically upon opening the fish (fish from one sampling site were exclusively examined histologically and immunohistochemically). Fish showing enlarged kidneys or kidneys with greyish focal to coalescing spots were graded as macroscopically positive for *T. bryosalmonae*.
b) One H&E stained kidney section from every fish was investigated for the presence of parasites within the interstitial tissue or within the tubules. Kidneys were graded as histologically positive when at least one parasite could be found. For the infection level a score from 0 (= no parasites) to 6 (at least 10 parasites per high power field (magnification 400x) was used.
c) Immunohistochemistry was applied on those sections where H&E histology revealed no clear results (e.g. proliferation of kidney but no parasites detectable, myxozoan parasites exclusively in tubules). Further, at sampling sites where no parasites had been found in any fish by examination of H&E stained kidney sections, three kidneys were randomly selected for immunohistochemical staining to verify the negative finding.

The final diagnosis "PKD-positive" based on the detection of *T. bryosalmonae* by histology or immunohistochemistry.

The site infection intensity B was calculated by the following formula (1):

$$B = \frac{sum\ of\ scores}{sample\ size} \quad (1)$$

Results

Sampling site specific results

PKD-positive fish were detected in 49 out of 91 rivers (54%) and at 62 out of 111 river sampling sites (56%). In three out of four farms PKD-positive fish were found. While in most positive sites at least some fish showed macroscopic symptoms typical for PKD, in fish from 12 positive sites no macroscopic symptoms were seen.

North of the Alps 44 out of 81 rivers (54%) and 56/100 sites (56%) were PKD positive (Fig. 1). The four farms examined, of which three were positive for PKD, are situated north of the Alps. In the central alpine valley (canton Valais) 3/7 sites (43%) were found to be positive. Each site represented a different river. South of the Alps, three out of four sites (75%) were

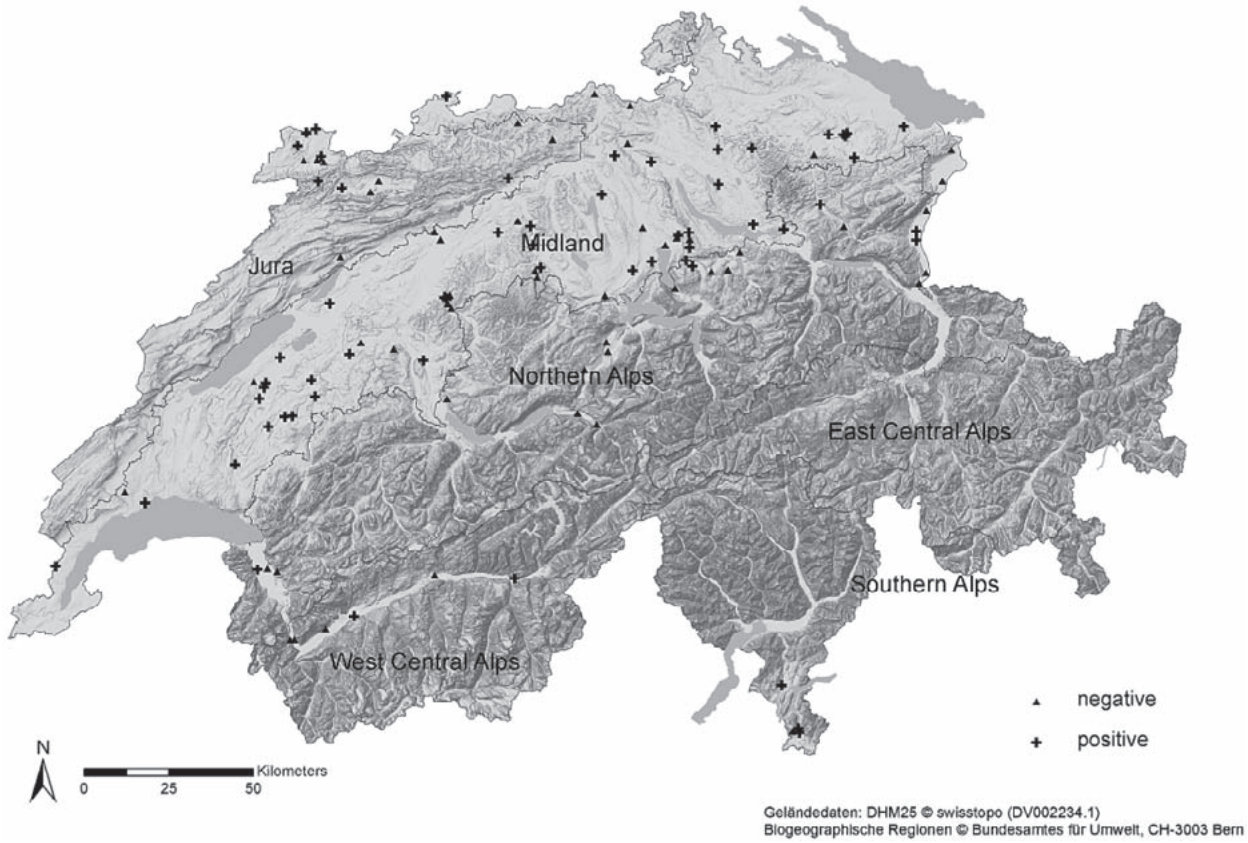

Figure 1. Distribution of sites with fish positive and negative for *Tetracapsuloides bryosalmonae* in a monitoring performed in 2004.

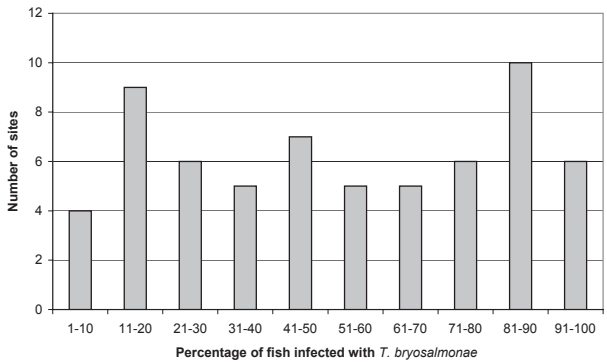

Figure 2. Number of sites in 2004 with a given prevalence of *T. bryosalmonae* infected fish. Only sites with fish positive for *T. bryosalmonae* included.

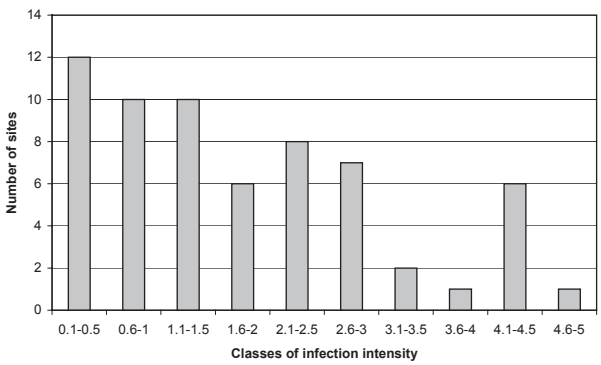

Figure 3. Distribution of investigated sites in 2004 according to mean infection intensities of fish by *T. bryosalmonae*. Only sites with fish positive for *T. bryosalmonae* are included.

diagnosed as PKD positive. Two of the positive sites were located in the same river. Therefore, in 67% of the rivers fish were affected.

At four of the 62 positive sites ≤10% of the analysed fish were infected by *T. bryosalmonae* (Fig. 2). This corresponds to 3.4% of all sites. In one of these sites, a positive result was based on a very low number of parasites found in one single fish by immunohistochemical staining only. At six sites, more than 90% of the examined fish proved to be positive and at four of these sites all fish were infected with *T. bryosalmonae*. Considering all sites with positive fish, the average prevalence was found to be 53% (minimum 5%, maximum 100%).

At PKD-positive sites values for the infection intensity were found to be between 0.1 and 3 (Fig. 3). Values higher than four were found in seven sites only. The mean infection intensity for all positive sites was 1.8 (minimum 0.1, maximum 5).

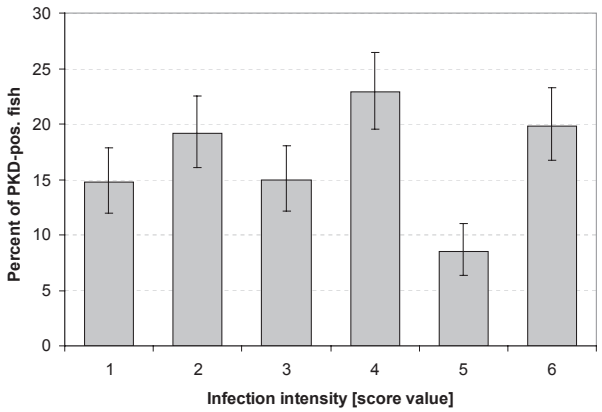

Figure 4. Prevalence of PKD-positive fish with a particular infection intensity ranging from 1 (= single parasites) to 6 (>9 parasites per high power field). Bars indicate the 95% confidence levels of the binomial distribution.

Fish specific results

T. bryosalmonae was detected histologically or by immunohistochemistry in 28% (590/2085) of fish. Of these, 2,048 fish were analysed by both, by macroscopical and histological methods. Thereby, 578 of these 2,048 fish were classified as PKD-positive. Of the positive fish, 157 showed neither clinical nor macroscopic alterations and were classified macroscopically as negative. Among the 226 fish that were not unequivocally identified as PKD-positive by H&E histology, 73 were found to be positive by immunohistochemical staining.

Half of the fish showed a moderate to high intensity of infection (score values of 4–6) (Fig. 4).

Many of the infected fish showed a prominent hyperplasia of the interstitial tissue accompanied by a severe granulomatous inflammation and fibroblast proliferation in the kidney. However, histology and immunohistochemistry did not identify *T. bryosalmonae* in all fish that showed marked pathological kidney alterations. The majority of such fish displaying kidney pathology but no parasites were caught at the end of September and in October.

In many fish with marked inflammatory reaction and development of fibrous tissue, the density of *T. bryosalmonae* cells was very patchy. Also marked differences in the size of parasites were evident, it ranged from 10 µm to 16.5 µm. In a number of fish, degenerated parasites, characterized by loss of structural integrity, were observed.

Discussion

PKD-positive fish were found in 56% of all sample sites investigated including rivers north and south of the Alps. The results confirm the findings of an earlier monitoring, which stated, that PKD occurs in a high prevalence in Swiss rivers (Wahli et al., 2002). The majority of positive sites in both monitorings has been found north of the Alps in the Swiss Midland (Fig. 5), though it has to be considered, that the majority of sites included in this study was as well located in the Midland. Nevertheless, there is some evidence that PKD can be found more frequently in areas with higher water temperatures in summer. This hypothesis has still to be confirmed. Similar investigations have been performed in rivers in England and Wales (Seagrave et al., 1981; Feist et al., 2002). The study by Feist et al. (2002) identified positive fish in five out of 14 examined rivers, a percentage clearly lower than in our investigations.

It has to be emphasized that our results may not reflect the true prevalence of PKD in Switzerland for two main reasons. 1.) Both of our investigations in Swiss rivers have been based on samplings performed only once per site thus reflecting the ad hoc situation. As shown by Okamura et al. (2001) the presence of *T. bryosalmonae* infected bryozoans shows considerable temporal variations. Whether this applies also to the presence of PKD in trout has still to be elucidated. Repetitive samplings at the same sites could clarify this question. 2.) The sample sites investigated in this study have not been randomly selected. Cantons and fisheries associations throughout Switzerland defined rivers and brooks where fish should be investigated. With this selection we wanted to expand on earlier studies (Wahli et al., 2002) and to gather knowledge on the status of rivers from which spawners are taken or fry are raised for restocking purposes. Nevertheless, the results demonstrate that *T. bryosalmonae* is widely distributed in wild fish of Swiss rivers.

The prevalence of PKD-positive fish at a site was normally >10%. This is in accordance with data found in England and Wales (Feist et al., 2002), where prevalences were between 11 and 43%. This indicates that fish are commonly diseased if *T. bryosalmonae* is present in the river. At only four sites the prevalence of PKD-positive fish was ≤10%. However, negative results have to be interpreted with caution because of the fair confidence level (82–85% for a minimum expected PKD prevalence of 10%) due to our target sample size of 20 fish to prove freedom from disease. In order to confirm the negative status of sites, it is planned for further studies to analyse a sample size of 60 fish per selected sites, which would allow us to prove freedom from disease with a confidence level of almost 95%. Those target sample sites will be chosen in rivers previously shown to be negative for PKD but likely to be a suitable environment for *T. bryosalmonae* according to the geographic situation and structure of the brown trout population.

The infection intensities ranged from very low (1) to high (6). Such variations, also found amongst individual fish within one sample site, might be influenced by dif-

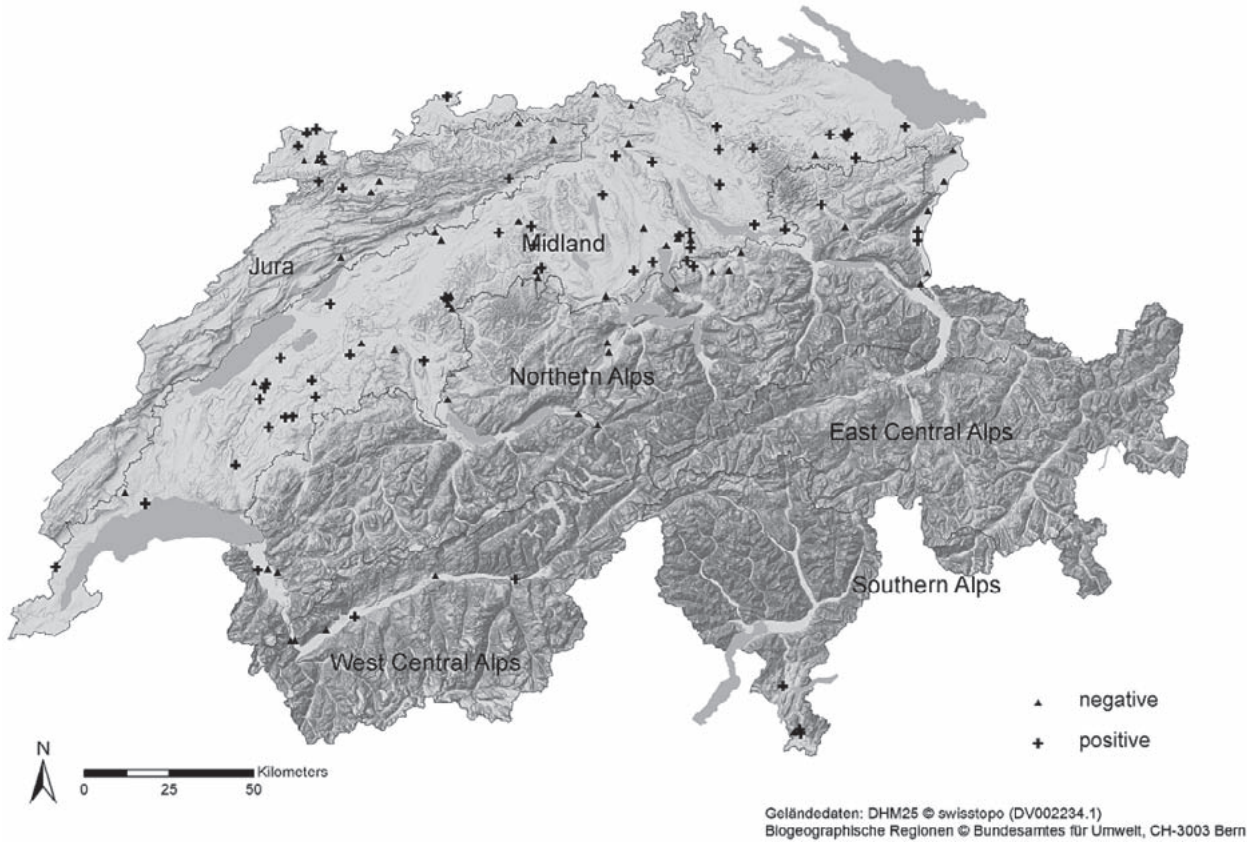

Figure 5. Distribution of sites with fish found positive for *Tetracapsuloides bryosalmonae* in two monitorings performed in 2000/2001 and 2004.

ferent factors. PKD is known to have a seasonal occurrence (Foott and Hedrick, 1987; Hedrick et al., 1993) with overt PKD developing mainly at temperatures of 15 °C or higher (Ferguson, 1981; Clifton-Hadley et al., 1986). In the present study, fish were sampled from August to October. In the vast majority of fish showing macroscopic symptoms the presence of parasites could be demonstrated either by histology or immunohistochemistry. Particularly in fish sampled towards the end of this period, kidney alterations typical for PKD (Ferguson and Needham, 1978), such as proliferation, inflammation and induration of fibrous tissue were present but no or only few parasites could be found. The pathology in these fish indicates a strong infection, however parasites might have left the host or been destroyed by the host's immune system at this time of the year. Therefore, to facilitate the diagnosis of PKD irrespective of the diagnostic method, samples should be taken at the peak period of the disease i.e. from July to mid-September. The lower frequencies for infection intensity classes of 3.1–3.5 and 3.6–4 might be due to the limited number of samples taken.

Spawners to produce broodstock for restocking measures are regularly taken from many of the rivers investigated. However, as vertical transmission has never been demonstrated, spread of PKD via eggs obtained from infected parent fish is unlikely. On the other hand, in some of the rivers and fish farms investigated, fry are raised for restocking measures. As certain infected salmonids can excrete small numbers of malacosporean like spores (Hedrick et al., 2004) an introduction of PKD into rivers so far free from *T. bryosalmonae* might occur if *T. bryosalmonae* infected young fish are stocked, though an infection of bryozoans by spores excreted from fish has not been achieved so far (Hedrick et al., 2004). It remains uncertain whether introduction of infected fry can spread the disease unless an infection of bryozoans by spores released from fish can be demonstrated.

Earlier studies in Switzerland pointed to a possible association between PKD and increased mortality in brown trout (Schmidt et al., 1999) and PKD was identified as a possible factor contributing to the declining brown trout catches in Switzerland. This hypothesis is supported by a negative correlation between the presence of PKD and the brown trout catches in five investigated cantons (Fischnetz, 2004; www.fischnetz.ch). Feist et al. (2002) showed a positive correlation between malacosporean infections and renal hypertrophy. These authors also suggested that infected fish are physically compromised. However, so far no published data showed

a clear correlation between the occurrence of PKD and a decrease in wild trout populations. Mortalities in wild fish populations directly attributable to a *T. bryosalmonae* infection have not been reported so far although an effect on a wild trout population has been shown for other myxozoans e.g. *Myxobolus cerebralis* (Hedrick et al., 1998; Modin, 1998).

Assessment of the impact of PKD on wild populations requires long-term, multidisciplinary, observational studies.

There is evidence that temperature plays a major role in the development of *T. bryosalmonae* in its bryozoan host (Gay et al., 2001) and the clinical disease of PKD is usually found during the warm water months of the year (Clifton-Hadley et al., 1986, Schmidt-Posthaus et al., 2001). There is some evidence for a general rise in water temperature in Switzerland (Hari et al., 2006) which might influence the disease development. Global changes leading to increasing water temperatures support the importance of follow-up studies of temperature dependent diseases (Fischnetz, 2004). A second possibly influential factor is water pollution (Schmidt et al., 1999; Schmidt-Posthaus et al., 2001). While Okamura et al. (2001) found bryozoans infected with *T. bryosalmonae* in all types of water irrespective of its quality, Hoffmann and Dangschat (1981) and El-Matbouli and Hoffmann (2002) suggested an influence of water quality on the course of PKD. Water contaminants can influence the disease via development of the parasite in the bryozoan host or in its fish host. Indeed, it is likely that the prevalence of PKD is highly influenced by environmental factors affecting the distribution of the invertebrate host (Okamura et al., 2001). Until now, there is little information on the distribution of bryozoans in Swiss rivers.

This study presents an extended data set on the presence of PKD in Swiss rivers. Time series and spatial analyses are necessary to investigate the importance of time-dependent risk factors, such as water temperature, and spatial variation of other environmental factors, respectively. Further research to investigate the occurrence of bryozoans and to identify factors influencing the development of PKD needs to be done. Such additional research is necessary for a better understanding of the epidemiology of PKD and its impact on wild brown trout populations.

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