

***Diphyllobothrium latum* (Cestoda: Diphylobothriidea) in perch (*Perca fluviatilis*) in three sub-alpine lakes: influence of biotic and abiotic factors on prevalence**

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ABSTRACT

In recent years, human diphylobothriosis has staged a comeback in Swiss, French and Italian sub-alpine regions. The main putative infective source of the causative agent (the tapeworm *Diphyllobothrium latum*) in these areas is perch (*Perca fluviatilis*). Therefore, the occurrence of *D. latum* in this fish species was investigated between 2005 and 2008 in the sub-alpine lakes Maggiore, Lugano and Geneva. Prevalence in fish of Lake Maggiore was 14% (n = 880). In Lake Geneva, 5.1% fillets (n = 532) were infected, whereas perch from Lake Lugano were free from the parasite. These results are discussed in relation to previous studies. Data on fish size and weight indicate that infection of perch by *D. latum* is independent of age and sex. Abiotic factors considered critical for *D. latum* life cycle (water temperature and oxygen concentration) characterize the three basins and were related to their infestation frequencies. The presence of this parasite was most likely favoured by warmer, well oxygenated waters. Previous studies indicate that the lake's trophic state (i.e. content of total phosphorus) influenced the availability of the first intermediate hosts (copepods) of some pseudophyllideans. In our study, no correlation was observed between the amount of phosphorus and the number of copepods in populations of zooplankton. Nevertheless, the trophic states of the three lakes seemed to affect the degree of infection in fish. In conclusion, at least in sub-alpine lakes, abiotic factors such as water temperature, oxygenation and trophic state seem to have an influence on maintaining or preventing perch infection with *D. latum*.

Key words: food-borne zoonoses, emerging infections, flatworms, helminths

1. INTRODUCTION

Human diphylobothriosis is a cosmopolitan fish-borne zoonosis caused by tapeworms of the genus *Diphyllobothrium* (Cestoda: Diphylobothriidea). The life cycle of the causative agent includes two intermediate hosts (copepods and fish) and a definitive host (a fish-eating mammal, mainly man). In the last 20 years, this disease has experienced an unexpected comeback in Swiss, French and Italian sub-alpine regions (Dupouy-Camet & Peduzzi 2004; Peduzzi 1990). Epidemiological monitoring and molecular identification of clinical cases in this area have revealed the presence of *D. latum* (the main autochthonous species infecting humans), as well as *D. nihonkaiense* and *D. dendriticum* (allochthonous species; Yéra *et al.* 2006; Wicht *et al.* 2007; Wicht *et al.* 2008; Paugam *et al.*, in press). Perch (*Perca fluviatilis*) was identified as a frequent host of plerocercoid larvae of *D. latum*. In the past years, a high prevalence in this fish has been demonstrated in several sub-alpine lakes: up to 33% of the population investigated was infected in Lake Orta (Northern Italy; Peduzzi & Boucher-Rodoni 2001); from 3.7% to 14.2% in the lakes of Bienne and Morat (Swiss Plateau; Golay & Mariaux 1995); 4-10% in Lake Geneva (Nicoulaud *et*

al. 2005); 7.8% in Lake Maggiore and 30% on average in Lake Como (Bonini *et al.* 1998; Gustinelli *et al.* 2007). However, some basins located in this region, such as lakes Varese and Lugano, were free from the parasite (Dupouy-Camet & Peduzzi 2004).

The presence of definitive hosts releasing eggs in water is obviously among the major requirements for maintaining *Diphyllobothrium* in a water system. This condition being satisfied in endemic sub-alpine areas, environmental factors might play a role in the development of ontogenetic stages of this tapeworm. Abiotic parameters such as water temperature, oxygen concentration (because eggs are aerobic) and light affect *Diphyllobothrium* embryonic development and the hatching of eggs (von Bonsdorff 1977). Moreover, for some freshwater fish parasites that depend on zooplankton for their development, the availability of these hosts has been linked to the lake's trophic state (determined by total phosphorus). Thus, oligotrophic conditions may lead to an increased ratio of copepods to cladocerans (Molzen 2005). On the contrary, eutrophic conditions would increase the proportion of cladocerans in zooplankton, reducing the availability of copepods as first intermediate hosts and therefore impairing the parasitic life cycle. The trophic state could therefore

represent another crucial component for the maintenance of *Diphyllbothrium* in a water body. To our knowledge, no previous studies that include these variables have been conducted on the occurrence of *Diphyllbothrium* in sub-alpine lakes.

The main objective of this study was to survey the occurrence of diphyllbothriosis in perch of the lakes Maggiore, Lugano and Geneva from 2005 to 2008. Fish samples were checked for the presence or absence of plerocercoid larvae, the identity of which was confirmed with molecular methods. Biotic (weight, size and sex of the fish) and abiotic factors affecting embryonation and egg hatching (water temperature and dissolved oxygen), were also taken into account. Trophic states of these lakes, evaluated by rates of total phosphorus in the epilimnion, were correlated with the occurrence of the parasite and the presence of its first intermediate hosts.

2. METHODS

2.1. Sampling

The study was carried out from July 2005 to June 2008. Samples of perch (*Perca fluviatilis*) were collected usually once in each season.

In Lake Maggiore, at least 200 individuals were collected each year. Fish were caught using gill nets with 28 × 28 mm mesh. Due to unfavorable weather conditions, sampling in winter 2007 could not be performed. Fifteen stations [Brissago, Porto Ronco, Ascona, Locarno, Muralto, Tenero, Foce Verzasca, Magadino, Vira Gambarogno, San Nazzaro, Gerra Gambarogno and Ranzo (Swiss part), Cannero, Baveno and Monvalle (Italian part)] were chosen to obtain a representative sample for the whole lake.

In Lake Geneva, a regular monitoring was carried out only from June 2006 to January 2008, as previous data were taken from Nicoulaud *et al.* (2005). Samples consisted mainly of perch fillets provided by local fishermen. Six stations were chosen in Petit Lac (Mies and Versoix, Canton Geneva, Switzerland) and in Grand Lac (Morges and Paudex in Canton Vaud, Switzerland; Séchex and Thonon-les-Bains in department of Haute-Savoie, France), which are characterized by different depths and intervals of water mixing, leading to different concentrations of oxygen and phosphorus (Lazzarotto *et al.* 2006).

Lake Lugano was sampled once in October 2005 and once in January 2006, and seasonally thereafter using gill nets with 32 × 32 mm mesh. Eight stations [Figino, Agno, Morcote, Brusino Arsizio and Capolago (South basin), Bissone and Gandria (North basin), and Ponte Tresa] were chosen in three compartments with different hydrological characteristics in Switzerland.

2.2. Fish analysis and parasite identification

Either whole fishes or fish fillets were investigated, depending on the type of samples provided by the fish-

ermen. Size (total length, measured from the extremity of the head until the inferior lobe of the tail) and sex were recorded for each fish. Both whole individuals and fillets were weighted.

Plerocercoids were isolated by visual inspection and successively identified by molecular methods. DNA was extracted from each larva following the Tissue Protocol of the QIAamp DNA Minikit (QIAGEN, Hombrechtikon, Switzerland). About 50 ng μL^{-1} DNA were tested by polymerase chain reaction using the Taq PCR Master Mix Kit (QIAGEN, Hombrechtikon, Switzerland), with specific primers for cytochrome c oxidase subunit I (Cox-1) complete gene amplification (Nakao *et al.* 2007). This mitochondrial gene is considered as the most discriminant within *Diphyllbothrium* (Isobe *et al.* 1998; Yéra *et al.* 2008). Amplicons were directly purified with NucleoSpin[®] Extract II Kit (Macherey-Nagel, Oensingen) and DNA was quantified with a ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). Sequencing reaction was prepared using the ABI PRISM[®] BigDyeTM Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Rotkreuz, Switzerland). After purification by filtration through MF[™] membrane filters 0.025 μm (Millipore, Zug, Switzerland), samples were loaded in an automatic sequencer (ABI PRISM[®] 310 Genetic Analyzer, Perkin Elmer, Rodgau-Jügesheim, Switzerland). Manually corrected sequences were aligned in a Neighbor-Joining tree (Kimura-2 parameters; bootstrap values for 1,000 replicates; MEGA version 3.0; Kumar *et al.* 2004) and analysed by a BLAST search (Altschul *et al.* 1990).

2.3. Statistical analyses

The prevalence of *Diphyllbothrium* in the samples was calculated for each lake. The age of the fishes was estimated from their weight and size according to Negri (2000) and Muus *et al.* (1971).

Missing weights of either fish or fillets were estimated using the formula:

$$\text{Fillet weight} = 1.915 + 0.213 \times \text{Fish weight} \quad (1)$$

derived from a regression analysis performed on the samples for which both fillets and whole fish weights were available ($r^2 = 0.95$).

The correlation between prevalence of infection and estimated weight, size, and sex of fish were checked graphically and using a chi square test. Perch show different linear and weight growth curves depending on their sex (Negri 2000). Correlation between fish weight and infection and between fish size and infection was therefore computed separately for female and male.

Monthly patterns of temperature, oxygen concentration and total phosphorus in the epilimnion (0-20 m depth), measured from January 2005 to December 2007 in all lakes (C.N.R.-I.S.E. 2008; CIPEL 2008; UPDA 2008), were analyzed graphically using scatterplot matrices.

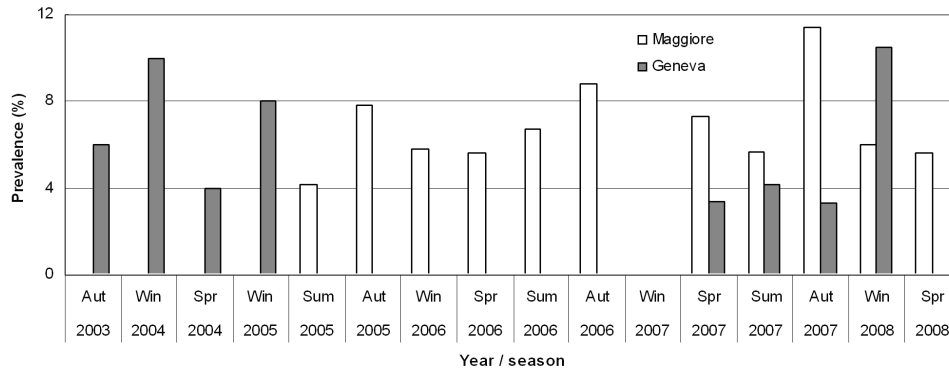


Fig. 1. Seasonal prevalence (%) of *D. latum* in perch fillets, sampled in Lake Maggiore and Lake Geneva from autumn 2003 to spring 2008 (data for 2003–2005 from Nicoulaud *et al.* 2005). Win: winter; Spr: spring; Sum: summer; Aut: autumn.

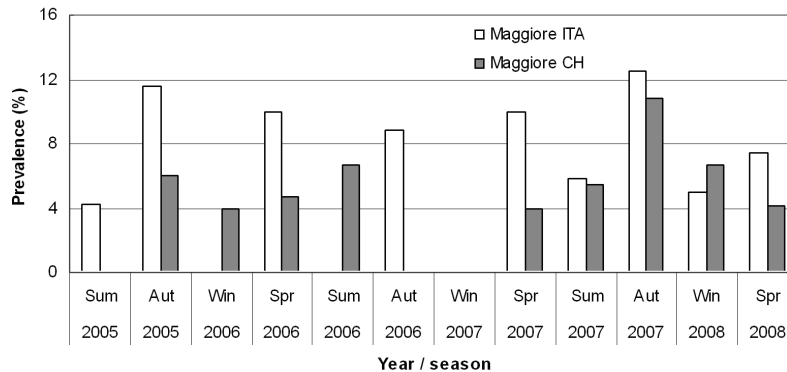


Fig. 2. Seasonal prevalence (%) of *D. latum* in perch fillets sampled in the Italian and in the Swiss portion of Lake Maggiore from summer 2005 to spring 2008. Win: winter; Spr: spring; Sum: summer; Aut: autumn.

For the same period, the correlation between the trophic state and the availability of copepods was checked for each water system. About 40 cyclopoid and calanoid species can be infected by *Diphyllbothrium* (Scholz *et al.* 2009). It has been suggested that their infection occurs mainly in littoral waters, where light and temperature conditions are more favourable to the development of coracidia (von Bonsdorff 1977). Most of the species present in the studied lakes (*Mixodiantomus laciniatus*, *Eudiaptomus padanus*, *Cyclops abyssorum*, *Megacyclops viridis* and *Mesocyclops leuckarti* in Lake Maggiore; *Eudiaptomus gracilis*, *Cyclops vicinus*, *Cyclops prealpinus* and *Macrocyclus albidus* in Lake Geneva; *Eudiaptomus gracilis*, *Cyclops abyssorum*, *Thermocyclops crassus* and *Mesocyclops leuckarti* in Lake Lugano) have been shown to be involved in the *D. latum* life cycle. As the parasite shows a quite broad specificity for its first intermediate hosts, we chose to pool them for the statistical analysis. The total phosphorus in the epilimnion (0–20 m) and the ratio of copepods to cladocerans (number of individuals per m³ sampled from 0–50 m; C.N.R.-I.S.E., 2008; CIPEL 2008; UPDA 2008) were compared by Analysis of variance (ANOVA).

All statistical calculations were carried out with SPSS version 14.0 (SPSS Inc. 2006).

3. RESULTS

3.1. Parasite identification

Molecular analysis of 94 parasites confirmed 100% identity of 1566 bp long sequences of the Cox-1 gene with *D. latum* (GenBank accession number AM778554: plerocercoid from perch of Lake Como, Italy and DQ985706: adult worm from a Russian patient). The similarity with a *D. latum* adult worm from another Russian patient (AB269325) was 99.9%.

The intraspecific variation consisted in a nucleotide substitution at position 90, where an adenine (A) replaced a guanine (G) in the sequence AB269325; this substitution is silent, because it does not affect the codon translation.

3.2. Prevalence

Plerocercoids of *D. latum* were detected in 14% of the perch population studied (n = 880) in Lake Maggiore, the highest prevalence being recorded in autumn, and 5.1% fillets (n = 532) in Lake Geneva, the highest prevalence being in winter (Fig. 1). Perch caught in the Italian part of Lake Maggiore were more infected than those sampled in the Swiss part (16.3% and 12%; Fig. 2). In Lake Geneva, the prevalence was

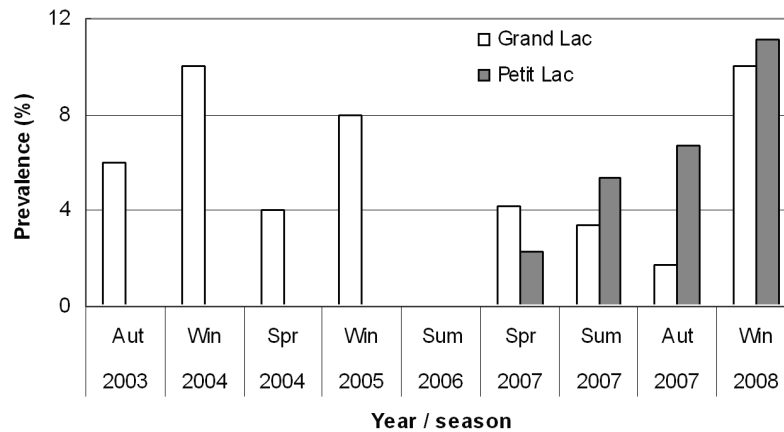


Fig. 3. Seasonal prevalence (%) of *D. latum* in perch fillets, sampled in Petit Lac and Grand Lac (Lake Geneva) from autumn 2003 to winter 2008 (data for 2003-2005 from Nicoulaud *et al.* 2005). Win: winter; Spr: spring; Sum: summer; Aut: autumn.

higher in Petit Lac (6.4% of the fillets) than in Grand Lac (4.5%, Fig. 3). No plerocercoid larvae were seen in 208 perch sampled in Lake Lugano.

With few exceptions, each fish/fillet was infected by only one plerocercoid. Infection by two plerocercoids was seen in 3 individuals from Lake Maggiore (Porto Ronco and San Nazzaro in spring 2007, and Monville in March 2008).

3.3. Biotic factors

In Lake Maggiore, the prevalence was 5.2% in males ($n = 328$) and 4.8% in females ($n = 800$). The presence of plerocercoids of *D. latum* was not related with the sex of the fish ($n = 1128$, Chi-Square = 0.094, $p = 0.762$). No correlation was seen between infection and the weight or size of fish, regardless of sex (data not shown).

In Lake Geneva no correlation was found between infection by *D. latum* and weight of the fillets ($n = 461$).

3.4. Abiotic factors

Respective monthly values of temperature ($^{\circ}\text{C}$), dissolved oxygen (mg L^{-1}) and total phosphorus ($\mu\text{g L}^{-1}$) in the epilimnion characterized each lake (Fig. 4; Tab. 1). Lake Maggiore showed a profile with low rates of total phosphorus (oligotrophy), the highest temperatures and high rates of dissolved oxygen. Lake Geneva (both Grand and Petit Lac) showed a similar profile of oxygen and temperatures, with moderately higher values of phosphorus (mesotrophy). Lake Lugano was characterized by high concentration of total phosphorus (eutrophy), lower temperatures and lower dissolved oxygen, showing a markedly different pattern compared to the two other basins.

No significant correlations were seen between the content of phosphorus and the availability of the first intermediate hosts. The amount of total phosphorus in the epilimnion had no influence on the number of copepods (linear regression analysis: Lake Maggiore: $p =$

0.771; Lake Geneva: $p = 0.788$; Lake Lugano: $p = 0.48$). No correlation was found between total phosphorus and ratio of copepods to cladocerans in lakes Maggiore (Pearson's correlation = 0.316, $p = 0.073$) and Geneva (Pearson's correlation = -0.253, $p = 0.178$); a positive correlation was seen only in Lake Lugano (Pearson's correlation = 0.414, $p = 0.012$).

4. DISCUSSION AND CONCLUSIONS

4.1. Prevalence

Between 2005 and 2008, *D. latum* plerocercoids were detected in perch in lakes Maggiore and Geneva. Fish in Lake Lugano was free from the parasite, confirming the results of previous studies (Dupouy-Camet & Peduzzi 2004).

The average prevalence in perch of Lake Maggiore (14%) has increased, especially if compared with previous surveys (7.8% in 1996; Bonini *et al.* 1998). The prevalence in fillets of Lake Geneva decreased slightly during the last 5 years: from 7% in 2003-2005 (Nicoulaud *et al.* 2005) to 5.1% in 2006-2008. However, the difference in sampling sites between the two periods (only Thonon-les-Bains in the previous survey, whereas 6 stations in this survey) did not allow us to draw any firm conclusion. In both lakes, the highest prevalence of infection was detected during the cold seasons (autumn and winter).

4.2. Biotic factors

The absence of correlation between the presence of the parasite and both linear and weight growth curves of perch of both sexes indicated that infection by *D. latum* of fish collected in Lake Maggiore occurred most likely throughout the whole fish life. The lack of any correlation between fillet weight and infection in Lake Geneva, even if the sex of fish could not be considered, led to the same conclusion. Estimated ages of infected samples from Lake Maggiore ranged from about 3 (16 cm length; 43.4 g; male caught in summer 2007) to more than 10 years (39 cm; 850.3 g; female caught in winter

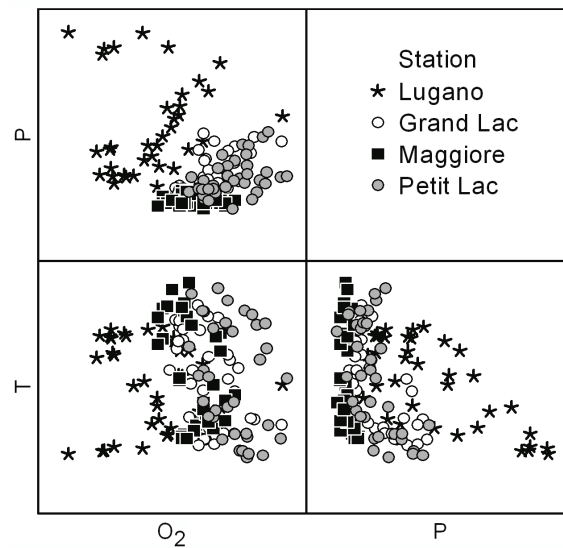


Fig. 4. Scatterplot matrix based on monthly values of temperature ($^{\circ}\text{C}$), dissolved oxygen (mg L^{-1}) and total phosphorus ($\mu\text{g L}^{-1}$) in the epilimnion (0-20 m) for the years 2005, 2006 and 2007, in lakes Lugano, Geneva (Petit Lac and Grand Lac) and Maggiore.

Tab. 1. Average seasonal temperatures ($^{\circ}\text{C}$) in lakes Maggiore, Geneva and Lugano, from 2005 to 2007. Win: winter; Spr: spring; Sum: summer; Aut: autumn.

LAKE	Win '05	Spr '05	Sum '05	Aut '05	Win '06	Spr '06	Sum '06	Aut '06	Win '07	Spr '07	Sum'07	Aut '07
L. Maggiore	6.9	11.6	18.6	13.4	7	10.5	17.5	13.2	8.1	13.8	18.7	9.2
L. Geneva	6	10.1	17.3	10.9	5.5	10.7	16.4	12.9	6.8	12.7	17.1	11.7
L. Lugano	5.8	11.9	15.8	10.9	5.2	11.7	15.6	11.4	7	12.7	15.5	10.5

2006-2007). Estimated ages of infected samples from Lake Geneva ranged from about 2 (21.3 g) to 5-10 years (199.5 g; both fish caught in winter 2007-2008). This demonstrates that the *D. latum* life cycle was ongoing in these basins, at least until 2004 in Lake Maggiore, and until 2006 in Lake Geneva.

No difference in *D. latum* prevalence was found between male and female.

4.3. Abiotic factors

Statistical analysis of temperature, dissolved oxygen and concentration of total phosphorus in the epilimnion indicated a correlation between the presence of *Diphyllbothrium* and the lake's profile. Lake Maggiore, where the average prevalence of *D. latum* was the highest (14% fish, i.e. 7% fillets) was characterized by warmer, well oxygenated water and oligotrophic conditions. Lake Geneva (5.1% fillets infected) was characterized by generally lower temperatures but higher oxygen concentration, and by mesotrophic conditions. As perch is a sedentary species (Muus *et al.* 1971), these data support the hypothesis that warm temperatures and high oxygen concentrations probably improve the conditions for the establishment of a parasitic life cycle. The abiotic profile of Lake Lugano (where *D. latum* was absent) was markedly different from that of the infected lakes, and was characterized by the lowest temperatures, the lowest dissolved oxygen and water eutrophy. For all lakes,

the presence of the first intermediate hosts was not correlated with phosphorus concentration.

Between 2002 and 2007, more than 260 human cases were reported on the Swiss and French shores of Lake Geneva while, from 2001, 40 cases were documented in the Insubrian region (lakes Maggiore, Lugano and Como; Wicht *et al.* unpublished). Such difference in the prevalence of human cases seems nevertheless to be unrelated to the variability of prevalence in local perch. These considerations suggest that the occurrence of *D. latum* in a lake is not only dependent on the presence of definitive hosts releasing eggs in water, and that abiotic factors could play a major role in maintaining (or in preventing) fish infection. For instance, Lake Lugano is located in an endemic area, but its perch are free from diphyllbothriosis.

Seasonality seems to be another parameter affecting infection frequencies. Under favorable environmental conditions (oxygen concentration: $8\text{-}12\text{ mg L}^{-1}$), transmission of *Diphyllbothrium* parasites to fish takes place quite rapidly (von Bonsdorff 1977). At $16\text{-}20\text{ }^{\circ}\text{C}$, eggs hatching takes 8-12 days; the development of proceroid larvae in copepods another 2-6 weeks (Bylund 2003). These data support the hypothesis that the best period for the transmission of *D. latum* to perch in lakes Maggiore and Geneva is summer. In fact, average oxygen concentrations in August from 2005 to 2007 were 9.2 mg L^{-1} in Lake Maggiore and 10.3 mg L^{-1} in Lake

Geneva (6.6 mg L⁻¹ in Lake Lugano; data not shown). Average temperatures in the same period were 19.3 °C in Lake Maggiore and 17.2 °C in Lake Geneva, but only 15.6 °C in Lake Lugano (data not shown). In addition, although adult perch (older than 3 years) are commonly considered ichthyophagous, it has been demonstrated that, in some Swiss and Italian lakes, individuals of any age feed on zooplankton during the summer (Brinker 2000; Negri 2000). Adults may switch to consumption of copepods mainly in July, when zooplankton is more abundant and its predation requires a minor energetic effort, compared to fish predation (Negri 2000). This would increase the possibility of a fish being infected by *D. latum* in the warm season. Moreover, in the last decades bleaks (*Alburnus* spp.), the main preys of perch in sub-alpine lakes, showed a marked decline, forcing perch to feed on alternative organisms (Peduzzi & Grimaldi 2007).

4.4. Limitations of the study

This study had some limitations. We tried to follow the same methods adopted in previous surveys in the same lakes, but some of the comparisons had to rely on data from previous studies, instead of novel controls. In addition, the monitoring in the individual lakes was not homogeneous. In lakes Maggiore and Lugano we employed the techniques described by Bonini *et al.* (1998) with regards to sample size, catch of whole individuals, filleting and visual inspection. In Lake Geneva fillets were provided directly by fishermen, as described by Nicoulaud *et al.* (2005), but with significant differences from the work described by these authors concerning sample size, sampling seasonality and number of stations. In addition, for logistic reasons, sampling could not be carried out consistently in the same periods in different years. The sometimes reduced sample size and missing data did not allow us to perform any confirmatory analyses.

4.5. Perspectives

D. latum is considered an indicator of faecal water pollution (von Bonsdorff 1977). In the future, it would be interesting to investigate the presence of infected fish near sewage treatment plants. In our study, the prevalence in the Italian part of Lake Maggiore was higher (16.3% perch infected) than in the Swiss part (12%). Data on the state of the lake indicate that in 2003 80% of the population not connected to sewage treatment plants was in Italy, 63% of which in Lombardy and 37% in Piedmont; conversely, more than 90% of the population was connected in Switzerland (CIPAIS, 2003). Nevertheless, the situation in Italy has markedly improved in the last years and the quality of waters has now greatly improved (CIPAIS 2003).

It is possible that climatic changes could favour *D. latum* life cycle by warming freshwater systems, thus increasing the percentage of infected fish (Patz *et al.*

2000; Marcogliese 2001; Sagua *et al.* 2001). This should also be investigated by future regular monitoring of endemic lakes located in endemic areas.

Other abiotic parameters are known to play a role on the occurrence of *D. latum* in water systems, such as salinity, pH, light intensity and pollutants (von Bonsdorff 1977). Their influence in the sub-alpine lakes deserves to be elucidated as well, and could help explain the differences in fish prevalence.

From a public health perspective, the presence of *D. latum* in perch in the studied lakes is of great importance, as the increasing popularity of raw fish dishes is thought to be linked to the re-emergence of human diphyllbothriosis in the sub-alpine area (Scholz *et al.* 2009). Health authorities should thus publish consistent and binding guidelines on the proper handling of raw fish as food source.

ACKNOWLEDGMENTS

We are grateful to all individuals who provided samples, in particular to Walter Branca, Ivano Conti, Armando Polli, Ermanno Gandolfi, Cooperativa Pescatori Professionali del Lago Maggiore, and to Daniel Gerdeaux. The collaboration by Eric Bernardi, Mauro Bordon, Laurence Calame-Haller, Manuel Marcu, Bruno Polli, Nicoletta Riccardi, Nadia Ruggeri, Jean Pascal Ubertini and Pietro Volta was crucial for the organization of the samplings. We gratefully acknowledge the invaluable help by Minoru Nakao and Tetsuya Yanagida (Department of Parasitology, Asahikawa Medical College, Hokkaido, Japan) in sequencing parts of the genome of the samples. Dr. Gary J. Samuels (USDA/ARS, Beltsville, MD, USA) critically read the manuscript and made helpful comments.

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Received: September 2008

Accepted: January 2009