Revue suisse de Zoologie (September 2019) 126(2): 219-225 ISSN 0035-418

Occurrence of *Dibothriocephalus latus* in European perch from Alpine lakes, an important focus of diphyllobothriosis in Europe

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Abstract: The broad fish tapeworm *Dibothriocephalus latus* (syn. *Diphyllobothrium latum*) is one of the most common causative agents of human diphyllobothriosis, a significant fish-borne parasitic zoonosis. In Europe, the occurrence of *D. latus* has been repeatedly reported in lakes of the Alps region, the Baltic region, Fennoscandia and Russia. Regular detection of *D. latus* plerocercoids in fish coming from different subalpine lakes linked with ongoing *D. latus* infection in humans indicates that Alpine region is a rather specific area from the medical, epidemiological and ecological point of view. Results from the examination of 688 European perch (*Perca fluviatilis*) from six subalpine lakes in Switzerland, France and northern Italy (Lakes Geneva, Neuchâtel, Biel, Como, Maggiore and Iseo) confirmed the ongoing occurrence of *D. latus* in the Alps region. The detected prevalence of *D. latus* in the studied Alpine lakes (2% in Lake Neuchâtel; 37.5% in Lake Biel; 6.4% in Lake Geneva; 22.8% in Lake Iseo [2018]; 12.8% in Lake Iseo [2017]; 15.2% in Lake Como; 16.7% in Lake Maggiore) was compared with previously published data. In addition, the importance of the Alpine lakes region and data on the epidemiology and ecology of *D. latus* related to subalpine lakes were discussed.

Keywords: Diphyllobothriosis - fish-borne zoonosis - plerocercoid - European perch - *Perca fluviatilis* - prevalence, genotyping.

INTRODUCTION

Studies on the geographic distribution of parasitic species are fundamental for understanding their circulation in the natural environment and for an assessment of endemic regions. In the case of parasites with zoonotic potential, knowledge on the distribution of a particular species is furthermore important for determining the potential risk for humans. Food-borne transmission of causative agents of parasitic zoonoses is considered to be one of the most frequent paths of infection. A special category of food-borne diseases are fish-borne parasitic zoonoses, characterized by their transmission via freshwater, brackish or marine fish.

Diphyllobothriosis, caused by so-called "broad tapeworms" or "fish tapeworms" of different genera of the order Diphyllobothriidea, is a significant fish-borne parasitic zoonosis responsible for about 20 million human infections worldwide (Chai *et al*., 2005). One of the most common causative agents of human diphyllobothriosis is the broad fish tapeworm *Dibothriocephalus latus* (Linnaeus, 1758) (Cestoda: Diphyllobothriidea), previously known under the scientific name *Diphyllobothrium latum.* Recent phylogenetic study of the order Diphyllobothriidea divided the polyphyletic genus *Diphyllobothrium* into two, *Diphyllobothrium* and the resurrected *Dibothriocephalus* (Waeschenbach *et al*., 2017) comprising seven species with *D. latus* as the type species.

The complex life cycle of this tapeworm involves two intermediate hosts (crustaceans and fish) and a definitive host (fish-eating mammals, including humans). Humans, probably the main definitive host maintaining the life cycle of the parasite (Golay & Mariaux, 1995), can be infected by the consumption of raw or undercooked fish fillets containing infective larval stages, plerocercoids. Diphyllobothriosis has been associated with eating habits, such as the consumption of salted or marinated fish fillets

Manuscript accepted 04.06.2019
DOI: 10.5281/zenodo.3463453

in the Baltic or Scandinavian countries and local raw fish specialities in the Alpine region (Switzerland, France and northern Italy), which represents the area with frequent or relatively frequent occurrence of diphyllobothriosis in humans (Golay & Mariaux, 1995; Desvois *et al.*, 2001; Peduzzi & Boucher-Rodoni, 2001; Terramocci *et al.*, 2001; Dupouy-Camet & Peduzzi, 2004; Yera *et al.*, 2008; Wicht *et al.*, 2010a, b). Infections in humans can be either autochthonous, or *D. latus* can be acquired during trips or stays abroad (imported cases). On the other hand, the direct detection of plerocercoids in the second intermediate fish host reflects the occurrence of the parasite in a particular natural environment. In Europe, the European perch (*Perca fluviatilis*) is the most suitable host of *D. latus*, while the Northern pike (*Esox lucius*) serves as a common paratenic host, and the burbot (*Lota lota*) plays a less important role in transmitting *D. latus* to humans (Gustinelli *et al.*, 2016). The occurrence of *D. latus* in fish has been frequently detected in lakes of the Alpine region, including northern Italy (Gustinelli *et al.*, 2016), Switzerland and France (Dupouy-Camet *et al.*, 2015). Moreover, regular detection of *D. latus* plerocercoids in fish was also confirmed in Russia (Klebanowski, 1985; Novak, 2012; Dugarov & Pronin, 2017), the Baltic region, in particular Estonia (Jõgiste & Barotov, 1993; Wicht *et al.*, 2010a, b), and Fennoscandia, including Finland (Andersen & Valtonen, 1992; Valtonen & Julkunen, 1995; Valtonen *et al.*, 1997), Norway (Vik, 1957) and Sweden (Akuffo *et al.*, 2003).

The Alpine region represents an important area from a medical and epidemiological point of view due to local eating habits and the relatively frequent detection of *D. latus* infection in humans. The rather constant prevalence and regular detection of *D. latus* plerocercoids in fish also make this region very interesting from an ecological point of view. The aim of this study was to provide the latest data on the occurrence of *D. latus* plerocercoids in European perch from different Alpine lakes to reveal the current situation of *D. latus* occurrence and to compare it with previously published data.

MATERIAL AND METHODS

A total of 688 European perch (*Perca fluviatilis*) originating from six Alpine lakes (Fig. 1) were examined for *D. latus* plerocercoids. Out of them, 156 fish originated from Lake Geneva (Switzerland/France), 50 from Lake Neuchâtel (Switzerland), 8 from Lake Biel (Switzerland), 46 from Lake Como (Italy), 48 from Lake Maggiore (Italy/Switzerland) and 380 from Lake Iseo (Italy). The examinations were carried out in March 2017 (Lakes Como, Maggiore and Iseo), May 2018 (Lake Iseo) and June 2018 (Lakes Geneva, Neuchâtel and Biel). The fish were caught by professional fishermen using gill nets and kept on ice until they were dissected. Fish were examined by parasitological necropsy focused on musculature and the peritoneal cavity (intestine, liver and other abdominal organs). In order to detect the presence of *D. latus* larvae in muscles, thin (approximately 5 mm) slices of fillets of whole fish were carefully examined by direct observation. The parasites found in host tissues were isolated by means of dissecting needles, checked under a stereomicroscope, placed in 0.9% NaCl solution and finally fixed in 96% molecular-grade ethanol for further molecular analyses.

For molecular genotyping of plerocercoids, genomic DNA of all individuals was isolated using the QIAamp®

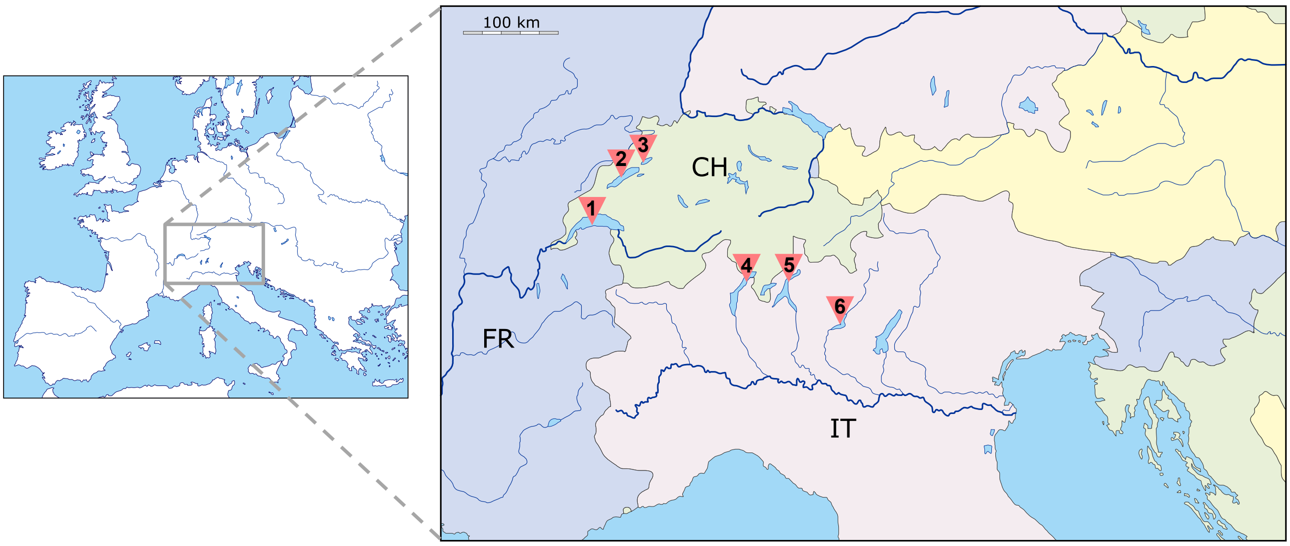

Fig. 1. Schematic representation of sampling sites of *Dibothriocephalus latus* from European perch (*Perca fluviatilis*) from the Alpine lakes region. (1) Lake Geneva; (2) Lake Neuchâtel; (3) Lake Biel; (4) Lake Maggiore; (5) Lake Como; (6) Lake Iseo. FR, France; CH, Switzerland; IT, Italy. Maps obtained from d-maps.com.

DNA Mini Kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer. *D. latus*-specific PCR was performed with the forward MulLat3 (GGGGTGTTACGGGTATTATACTC) and reverse MulRevCom (ATGATAAGGGAYAGGRGCYCA) primers designed for amplification of a partial (437 bp) fragment of mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1 mt DNA), according to Wicht *et al*. (2010b). The PCR products were visualized on 1.5% agarose gel and purified using exonuclease I and shrimp alkaline phosphatase (Werle *et al*., 1994). Sequencing was performed using the automatic genetic analyzer Applied Biosystems 3130xl (Applied Biosystems, Foster City, California, USA) and the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). Contiguous sequences were assembled and inspected for errors using Geneious (version 10.0.5, Biomatters, Auckland, New Zealand). The newly obtained *cox*1 sequences were compared with respective sequences of *D. latus* available in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

Table 1 summarizes data on the fish examined and the prevalence detected in each particular lake. The lowest prevalence (2%) was detected in Lake Neuchâtel. The highest prevalence (37.5%) was observed in Lake Biel; however, this could be influenced by the rather low number (no = 8) of fish examined. As for the other four lakes, the prevalence varied from 6.4% (Lake Geneva) up to 22.8% (Lake Iseo; 2018). The prevalence was mostly in the range of 10-20%, in particular 12.8% (Lake Iseo; 2017), 15.2% (Lake Como) and 16.7% (Lake Maggiore). Each fish was mainly infected by a single plerocercoid in the musculature, predominantly in dorsal muscles. In Lake Geneva, three fish were infected with two larvae and one perch harboured five plerocercoids.

Altogether, 108 plerocercoids were obtained from 688 European perch from the six studied Alpine lakes; all of them were subjected to molecular genotyping. Sequences of a 430 bp mitochondrial *cox*1 fragment of all individuals were 100% identical, and no intraspecific genetic diversity was observed. Comparison of the newly obtained data with the sequences deposited in the GenBank revealed 100% identity with *D. latus* from European perch from Switzerland (GenBank Accession numbers FM209180-1, GU997614, AY972071, DQ768197) and Italy (GU997613, KU341699, KU341702, KU341709, KU341711-2, KU341716), as well as with *D. latus* from Northern pike (KU341707 and KU341714) and burbot (KU341705) from Italy.

DISCUSSION

Current data on the occurrence of *D. latus* in perch from Lakes Como, Iseo, Maggiore, Geneva, Biel and Neuchâtel confirmed the presence of this medically important causative agent of diphyllobothriosis in the Alpine region, corresponding well to previously published results (Bouvier *et al*., 1963; Golay & Mariaux, 1995; Peduzzi & Boucher-Rodoni, 2001; Dupouy-Camet & Peduzzi, 2004; Nicolaud *et al*., 2005; Wicht *et al*., 2009a, b; Prearo *et al*., 2013; Dupouy-Camet & Yera, 2015; Dupouy-Camet *et al*., 2015; Gustinelli *et al*., 2016). These surveys were predominantly focused on examinations of European perch, the most suitable host of *D. latus* (Table 1). Hence, the data provided in the current paper were based only on examinations of perch, and no other fish species were investigated.

As has been observed previously, the majority of plerocercoids collected from perch were found in fillets, while only a few of them were collected from the serosa (Gustinelli *et al*., 2016). The location of plerocercoids in the visceral cavity also depends on the size of the perch. According to the personal experience of one co-author (A. Gustinelli), perch of larger size can ingest smaller infected perch, thus becoming a paratenic host, similarly to burbot and pike. In the current study, all plerocercoids were found in perch fillets; no larvae were detected in the peritoneal cavity or internal organs. The length of the perch examined ranged between 100-300 mm; however, most of the fish were around 150 mm in length, which may explain the absence of plerocercoids in the serosa.

Most studies on *D. latus* prevalence in fish have focused on Lake Geneva, shared between Switzerland and France, where the prevalence of *D. latus* in perch ranged between 4.0-29.2% (Dupouy-Camet & Peduzzi, 2004; Nicolaud *et al*., 2005; Wicht *et al*., 2009b; Dupouy-Camet & Yera, 2015; Dupouy-Camet *et al*., 2015). In contrast, the oldest data on the prevalence of *D. latus* in fish in Lake Geneva provided significantly higher values, in particular 95% in burbot and 58% in perch (Ketchekian, 1909 cited in Bouvier *et al*., 1963). These findings indicated the specific dynamics of the occurrence of *D. latus* in the Alpine region. Diphyllobothriosis caused by *D. latus* was a widespread fish-borne zoonosis at the beginning of the past century; however, it virtually disappeared later on (Gustinelli *et al*., 2016). Since the early 1980s, especially after year 2000, diphyllobothriosis has shown a comeback in some subalpine areas, particularly on the shores of the great subalpine lakes (Wicht *et al*., 2009a, b).

Currently, the detected prevalence of *D. latus* in perch from Lake Geneva was 6.4%, which fits into the most frequent and previously published values (4.0-12.0%) (Table 1). A rather high prevalence (22.2-28.6%) has been detected in burbot, and even a 100% prevalence was documented in pike (Dupouy-Camet & Yera, 2015; Dupouy-Camet *et al*., 2015). In general, pike seems to be the host with the highest detected prevalence of *D. latus*, with 12.5% in Lake Morat and 14.3% in Lake Biel, up to very high values of 71.4% (Lake Iseo), 84.2% (Lake Como) and 100% in Lakes Maggiore and Geneva (see Table 1 for references).

In the current study, the detected prevalence of *D. latus*

Table 1. Summary of literature data and current results of prevalence of *Dibothriocephalus latus* plerocercoids in European perch (*Perca fluviatilis*), pike (*Esox lucius*) and burbot (*Lota lota*) from the Alpine lakes in Italy (Lakes Como and Iseo), Italy/Switzerland (Lake Maggiore), Switzerland/France (Lake Geneva) and Switzerland (Lakes Biel, Morat and Neuchâtel).

Country	Locality	Host	No. of fish/ fillets examined	Prevalence (%)	Reference
Italy	Lake Como	European perch	609	30.0	Wicht *et al*., 2009a
			390*	28.7	Prearo *et al*., 2013
			426	25.4	Gustinelli *et al*., 2016
			46	15.2	current study
		Pike	19	84.2	Gustinelli *et al*., 2016
		Burbot	55	3.6	Gustinelli *et al*., 2016
	Lake Iseo	European perch	458	7.6	Gustinelli *et al*., 2016
			148	12.8	current study (2017 sampling)
			232	22.8	current study (2018 sampling)
		Pike	7	71.4	Gustinelli *et al*., 2016
		Burbot	26	3.8	Gustinelli *et al*., 2016
Italy/Switzerland	Lake Maggiore	European perch	309	7.8	Peduzzi & Boucher-Rodoni, 2001
			880**	14.0	Wicht *et al*., 2009b
			635	6.6	Gustinelli *et al*., 2016
			48	16.7	current study
		Pike	1	100	Gustinelli *et al*., 2016
Switzerland/France	Lake Geneva	European perch	50*	8.0	Dupouy-Camet & Peduzzi, 2004
			50*	12.0	Dupouy-Camet & Peduzzi, 2004
			50*	4.0	Nicolaud *et al*., 2005
			50*	6.0	Nicolaud *et al*., 2005
			50*	8.0	Nicolaud *et al*., 2005
			50*	10.0	Nicolaud *et al*., 2005
			532**	5.1	Wicht *et al*., 2009b
			24*	29.2	Dupouy-Camet *et al*., 2015
			25*	28.0	Dupouy-Camet & Yera, 2015
			156	6.4	current study
		Pike	160	12.5	Bouvier *et al*., 1963
			6*	100	Dupouy-Camet *et al*., 2015
			7*	100	Dupouy-Camet & Yera, 2015
		Burbot	7*	28.6	Dupouy-Camet *et al*., 2015
			9*	22.2	Dupouy-Camet & Yera, 2015
Switzerland	Lake Biel	European perch	81	3.7	Golay & Mariaux, 1995
			8	37.5	current study
		Pike	7	14.3	Golay & Mariaux, 1995
	Lake Morat	European perch	19	5.3	Golay & Mariaux, 1995
		Pike	8	12.5	Golay & Mariaux, 1995
	Lake Neuchâtel	European perch	50	2.0	current study
		Pike	26	11.5	Bouvier *et al*., 1963

* indicates examination of European perch fillets; ** either whole fish or fish fillets were investigated depending on the type of samples provided by the fishermen.

in perch from Lake Como was the lowest (15.2%) when compared with previously reported data (Table 1). In three other lakes (Iseo, Maggiore and Biel), the prevalence in perch revealed the highest values in comparison with previous data (Table 1). Since only eight fish were currently examined in Lake Biel, and a different sample size of examined fish was analysed in the present work and previously published surveys, it is difficult to compare data on prevalence and make reliable conclusions.

Besides the Alpine lakes, the presence of *D. latus* has also been confirmed in fish in Russia, the Baltic region, and Fennoscandia (see the above references in the Introduction). However, diphyllobothriosis seems to be strongly reduced or even to have disappeared in many of these localities (Scholz & Kuchta, 2016), which may have several explanations. In the past, *D. latus* occurred in a particular locality, but due to some environmental/ biological changes preventing the maintenance of the parasite life cycle, it has disappeared from the region. Another explanation for the lack of data on *D. latus* occurrence in previously endemic areas, such as the Baltic region and Fennoscandia, are altered eating habits of humans, resulting in a decrease of diphyllobothriosis to the point that it is no longer a serious medical problem. As a consequence, physicians and parasitologists can pay lower attention to this parasitosis.

In the subalpine territory, humans seem to be the most suitable host of *D. latus* (Wicht *et al*., 2009b) and probably serve as the only definitive host maintaining the life cycle of the parasite (Golay & Mariaux, 1995). Wild or domesticated carnivores may play only a minor role in the continuation of the cycle (Dupouy-Camet & Peduzzi, 2004). Although knowledge on the ecology and epidemiology of helminths shared by wild carnivores in Europe is limited, wild felids can contribute to environmental contamination and maintenance of the life cycle of *D. latus* (Otranto *et al*., 2015; Zottler *et al*., 2019). However, the latest examination of 168 stray cats in different regions in Switzerland revealed the presence of *D. latus* eggs only in one faecal sample (Zottler *et al*., 2019).

The presence of *D. latus* in perch in Alpine lakes is of great importance from a public health perspective, as the increasing popularity of raw fish dishes is thought to be linked to the re-emergence of human diphyllobothriosis in the subalpine area (Wicht *et al*., 2009b). Plerocercoids may survive in perch from several months up to a few years (Dick *et al*., 2001; Dick, 2007), and since they mainly prefer dorsal muscles (Prearo *et al*., 2013), they can be easily missed during food preparation (Jackson *et al*., 2007). Therefore, it is not surprising that human diphyllobothriosis in the Alpine lakes has corresponded to the infection of perch with *D. latus* plerocercoids (Wicht *et al*., 2007), and the subalpine area has been the site of several episodes of human infections (Gustinelli *et al*., 2016). Ineffective sewage treatment systems, which lead to the contamination of lakes by cestode eggs shed by infected humans, is the second main risk factor for the persistence of diphyllobothriosis in subalpine lakes (Gustinelli *et al*., 2016). In addition, faecal pollution of lakes by professional and leisure fishing on and around lakes can also be considered as one of the important factors in the continuation of the life cycle (Dupouy-Camet & Peduzzi, 2004). Therefore, preventive measures, sanitary precautions and healthcare education concerning safe raw fish preparation have been permanently important and should also continue in future.

Ongoing epidemiological and ecological monitoring of diphyllobothriosis in the Alpine region has provided the latest data and dynamics on the occurrence of this fish-borne zoonosis. As for other European regions, up-to-date surveys are necessary for accurate knowledge on human diphyllobothriosis and the circulation of *D. latus* in the natural environment. Special attention should be paid to localities with previous findings of the tapeworm based only on morphology. Modern differentiation techniques and the latest knowledge on ecology and distribution of *D. latus* should be applied in future studies on this medically important and biologically interesting tapeworm.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Mrs Janik Pralong from the Muséum d'histoire naturelle, Geneva, Switzerland and Dr Roman Kuchta, Institute of Parasitology, České Budějovice, Czech Republic for their valuable assistance during field work. The authors are grateful to the reviewers for their valuable comments and suggestions, which improved the manuscript. The work was financially supported by the Slovak Research and Development Agency under contract APVV-15-0004, the Slovak Grant Agency VEGA no. 2/0134/17 and by the Research & Development Operational Programme funded by the ERDF: Environmental protection against parasitozoonoses under the influence of global climate and social changes (code ITMS: 26220220116; rate 0.2).

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