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Prevalence of *Diphyllobothrium latum* (Cestoda: Diphyllobothriidae) plerocercoids in fish species from four Italian lakes and risk for the consumers

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Summary

In recent years there has been a re-emergence of diphyllobothriasis by *Diphyllobothrium latum* (Cestoda: Diphyllobothriidae) in Italy, France and Switzerland, where in the past this fish-borne zoonosis was widespread and then virtually disappeared. A change in eating habits such as the consumption of raw/undercooked freshwater fish, has led to an increased risk for consumers of ingesting infective larvae of *D. latum*. A survey on the factors responsible for the re-emergence of human diphyllobothriasis in Italy was carried out from March 2013 to December 2014. The aim of this study was to assess the diffusion of *D. latum* plerocercoids in the fish populations of the sub-alpine lakes of Maggiore, Como, Iseo and Garda, updating the scarce historical data and assessing a preliminary "risk level" of the lacustrine environments and fish species under investigation. A total of 2228 fish belonging to 5 species, 690 from Lake Maggiore, 500 from Lake Como, 655 from Lake Iseo and 383 from Lake Garda Lake were submitted to parasitological examination. The presence of *D. latum* plerocercoid larvae was detected in 6.6%, 25.4% and 7.6% of perch (*Perca fluviatilis*) from Lakes Maggiore, Como and Iseo respectively. The parasite was also present in pike (*Esox lucius*) with prevalence values ranging from 71.4 to 84.2% and in 3.6-3.8% of burbot (*Lota lota*) from Lakes Iseo and Como. Fish from Lake Garda were negative as well as sampled whitefish (*Coregonus lavaretus*) and shad (*Alosa fallax lacustris*). The results of this survey showed a widespread presence of *D. latum* plerocercoid larvae in Maggiore, Como and Iseo fish populations. Urban fecal contamination of water is still a key issue to be resolved, together with the improvement of communication with consumers regarding the best dietary habits and the most effective processess of parasite inactivation, required for the consumption of raw/undercooked fish caught in high-risk areas.

Key words: *Diphyllobothrium latum;* plerocercoid larvae; fish-borne zoonosis; Italy.

1. Introduction

Diphyllobothriasis by *Diphyllobothrium* spp. (Cestoda: Diphyllobothriidae) is a cosmopolitan disease affecting man and other fish-eating mammals as definitive hosts and involving fish as intermediate hosts of infective plerocercoid larvae. Despite its impact on human health, the disease is in progressive decline in many countries yet there are still countries showing a parallel increase (Dick, 2008; Dick et al., 2001; Scholz et al., 2009).

Diphyllobothrium latum is the most widespread species and since its discovery back in 3600 B.C. (Dommelier-Espejo, 2001) the subalpine area between France, Italy and Switzerland has been the scene of a number of episodes of human infections.

The zoonotic problem still seems far from being eradicated judging by the various cases observed since 2000 (de Marval et al., 2013; Wicht et al., 2007) after a long period of rather sporadic reports. Particularly in the Italian context, the occurrence of human infections during the first half of the 20th century, convinced the legislators to have *D. latum* plerocercosis listed as a notifiable disease in the Veterinary Regulation on Control Measures of Transmissible Animal Diseases (Regolamento di Polizia Veterinaria n. 320/1954). Only a few sporadic cases were observed in the following time span (Chiodera et al., 1986; Grassi and De Carneri, 1969; Pampiglione and Di Guardo, 1968; Scolari, 1955) and diphyllobothriasis has long been considered a public health issue of minor importance.

In recent years, there has been an increasing occurrence of human diphyllobothriasis in the Alpine areas of Switzerland, France and Italy (Dupouy-Camet and Peduzzi, 2004; de Marval et al., 2013; Terramocci et al., 2001; Vaiani et al., 2006; Wicht et al., 2007b). The case histories of the patients affected invariably reported the consumption of raw or

78 undercooked local lake fish (European perch, *Perca fluviatilis*, and whitefish,
79 *Coregonus* spp.).
80 All the human cases reported by these countries have been attributed to *D. latum*, with
81 the exception of some cases of *D. nihonkaiense* from raw Pacific salmon
82 (*Oncorhynchus keta*) imported from North America (Wicht et al., 2007a) and of *D.*
83 *dendriticum*, probably underdiagnosed in the past and acquired from raw fish eaten
84 abroad (Kuchta et al., 2013; Wicht et al., 2008).
85 The scientific community appears to widely accept the fact that the new emergence of
86 human diphyllobothriasis is mainly due to the recent changes in the eating habits of the
87 local populations (Chai et al., 2005; Scholz et al., 2009), with the appearance of cold
88 smoked, marinated or raw fish products, such as *carpaccio*. Moreover, the complexity
89 of the life cycle of *D. latum* and its various survival and transmission strategies, has led
90 to its persistence in the sub-alpine environment due to a number of additional factors
91 which are difficult to assess individually and require a holistic approach.
92 Due to this re-emergence, the urgent need to determine consumer risks by carrying out
93 epidemiological studies on this and other fish-borne zoonoses has been strongly
94 emphasized by the "Scientific Opinion on Risk Assessment of Parasites in Fishery
95 Products" published in 2010 by the European Food Safety Authority (EFSA).
96 The main aim of this study, carried out in the framework of a research project funded by
97 the Italian Ministry of Health (Ricerca Finalizzata RF-2010-2311360), is to provide a
98 preliminary answer to the EFSA call by studying the current diffusion of plerocercosis
99 by *D. latum* in fish species of Lakes Maggiore, Como, Iseo and Garda in order to
100 update the epidemiological situation and to identify the main risk factors for the
101 transmission of the parasite to consumers.

1. Materials and Methods

From March 2013 to December 2014 a parasitological survey was carried out on 2228 fish, 690 from Lake Maggiore, 500 from Lake Como, 655 from Lake Iseo and 383 from Lake Garda. The lakes under study are the largest glacial lakes in the sub-alpine area of Northern Italy (Fig. 1). The sampling sites were selected with the aim of monitoring the largest part of the lake surface area depending on fishermen availability.

The fish species examined were European perch (*Perca fluviatilis*), pike (*Esox lucius*), burbot (*Lota lota*), whitefish (*Coregonus* spp.) and shad (*Alosa fallax lacustris*) (Table 1). The sample size has been calculated for perch considering an expected prevalence of 5%±2.5% (CI 95%). A sample size of at least 281 fish to be examined for each lake has been considered. The other fish species have been sampled when available.

The fish were caught by professional fishermen using gill nets, immediately stored in cold boxes and then transported to the Laboratory of Fish Pathology, Department of Veterinary Medical Science, University of Bologna, Italy. All of the fish were weighed, measured and subjected to parasitological examination according to Roberts et al. (2012). In order to detect the presence of plerocercoids larvae of *D. latum*, the abdominal cavity and fillets were carefully examined by direct observation and/or candling (visual inspection as indicated by EU regulation, in particular Regulation (EC) No 853/2004 and Regulation (EC) No 2074/2005.

The plerocercoid larvae were isolated from the host tissue by means of dissecting needles, and were then counted and fixed in 70% ethanol. The morphological identification of the larvae was carried out in accordance with the key provided by Andersen and Gibson (1989).

126 Prevalence (P), Intensity (mean intensity, MI) and Mean Abundance (A) were
127 calculated according to Bush et al. (1997) by QP 3.0 software version 2.0 (Ròzsa et al.,
128 2000). Exact binomial confidence intervals 95% were calculated using STATA® 11.2.
129 Total DNA was extracted with a commercial kit (PureLink Genomic DNA Mini Kit,
130 Invitrogen) from 20 plerocercoid larvae collected from perch, pike and burbot caught in
131 the 3 sampling sites. The DNA were subjected to multiplex PCR of the cytochrome *c*
132 oxidase 1 (COI mtDNA) following the protocol reported by Wicht et al. (2010), which
133 allows for the simultaneous amplification of *D. latum* (437 bp), *D. pacificum* (318 bp),
134 *D. nihonkaiense* (1232 bp) and *D. dendriticum* (727 bp), the most common species
135 infecting humans. Amplification products were resolved on a 1% agarose gel stained
136 with SYBR Safe DNA Gel Stain in 0.5X TBE (Molecular Probes - Invitrogen,
137 Carlsbad, CA). The bands were excised and purified by NucleoSpin Gel and PCR
138 Clean-up (Mackerey-Nagel GmbH & Co. KG, Neumann-Neander-Str., Düren
139 Germany) and sequenced with an ABI 3730 DNA analyzer at StarSEQ GmbH (Mainz,
140 Germany). Following assembly with Vector NTI AdvanceTM 11 (Invitrogen, Carlsbad,
141 CA), the sequences were published in GenBank (KU341698-KU341717).
142
143 **2. Results**
144 Results of the parasitological analyses in the examined fish species are presented in
145 Table 2. Perch, pike and burbot have been foun infected by plerocercoid larvae of
146 *Diphyllobothrium* sp. while whitefish and shad were always negative.
147 Concerning the lacustrine environments, results are displayed in Table 3, with similar
148 positivity in perch from Lakes Maggiore and Iseo and a higher prevalence in Como
149 Lake, while Lake Garda was always negative (Table 3).

Most of the plerocercoid larvae collected from perch was found in the fillet while fewer were detected in the serosa (219 *vs* 4). An opposite trend was observed in pike (68 larvae in the fillet *vs* 467 in the serosa) and burbot (no larvae in the fillet *vs* 6 in the serosa).

The morphological characteristics of all the plerocercoid larvae were consistent with *D. latum*. The multiplex PCR of the COI gene confirmed that the plerocercoid larvae belonged to *D. latum* (437 bp). The sequences obtained from the 20 larvae were identical to one other and the BLAST search revealed 99-100% identity with *D. latum*.

3. **Discussion**

The zoonotic parasite *D. latum* is widely diffused in perch, pike and, to a lesser extent, in burbot from sub-alpine Italian lakes.

Lake Como showed the most worrying prevalence values compared to Lakes Iseo and Maggiore (25.4% *vs* 7.6% and 6.6%, respectively), meaning that 1 in 4 perches was parasitized by a plerocercoid larval stage. The prevalence values in perch caught in Lakes Como and Iseo substantially confirm previous data from literature (Borroni and Grimaldi, 1973; Gustinelli, 2008; Parona, 1887; Prearo et al., 2013; Scolari, 1955; Wicht et al., 2009), while the fish caught in Lake Garda were always negative as already reported by Parona (1887) and Scolari (1955). On the contrary, Borroni and Grimaldi (1973) found 0.4% of positivity in perch, but the fish examined were bought in the local fish market so it was impossible to trace the origin of the positive fish (Grimaldi pers. comm.).

Concerning infection intensity, the values obtained during this survey are similar to the scarce amount of data reported in past research studies (Gustinelli, 2008; Borroni and Grimaldi, 1974). In general infected perch showed 1 plerocercoid larva/fish, suggesting a high success rate of colonization and survival capability of this parasite when ingested by a suitable definitive host.

In most of the perch examined, the larvae were in the fillets, as typically reported for this fish species. An interesting finding was the unusual presence of plerocercoids in the muscles of the pikes, probably due to a residual migratory attitude of a few larvae. This could represent an additional risk factor for the consumer. However, in Italy the transmission of *D. latum* plerocercoids from pikes to humans is probably due to the accidental ingestion of the parasite during fish evisceration, as this fish species is not generally eaten raw or undercooked. Many fish species have been described as being suitable second intermediate hosts of *D. latum* in various geographical areas. Our data confirm that in continental Europe at least three fish species are suitable hosts for the development/survival of the plerocercoid larvae of *D. latum*: European perch (*Perca fluviatilis*), pike (*Esox lucius*) and burbot (*Lota lota*). For this study the fish species were primarily selected according to their importance in the transmission of the plerocercoid larvae of *D. latum*: the European perch has been confirmed as being the most suitable second intermediate host of *D. latum*, and the pike as common paratenic host. According to the results obtained burbot, plays a less important role in transmitting *D. latum* to humans. Whitefish (*Coregonus* spp.) and shad (*Alosa fallax lacustris*) were always negative for plerocercoids thus confirming they are not suitable hosts for *D. latum*. Currently they appear to play a negligible role in transmitting *D. latum*

196 plerocercoids to humans which is an interesting result considering their importance for
197 the economy of local professional fishermen.
198 It is important to note that unlike wild fish, no human infestations of *D. latum* have
199 currently been linked to farmed freshwater fish. Although few data are present in
200 literature (EFSA, 2010) and large scale surveys on farmed fish are required, artificial
201 feeding used in intensive fish farms should reduce the possibility of acquiring infected
202 copepods from the environment.
203 In Italy in 1894 Parona attributed the high diffusion of plerocercoid larvae of *D. latum*
204 in fish from sub-alpine lakes to the higher frequency of human diphyllobothriasis
205 observed in people living along the shores of these lakes. In 1901 Perroncito pointed out
206 the huge diffusion of *Diphyllobothrium latum* (*Bothriocephalus latus*) in western
207 Switzerland, estimating that one forth of the people living in Geneva could be infected.
208 After a long period of sporadic reports, over the last few decades new outbreaks of
209 human infection have been observed in some sub-alpine lakes in Italy (Lakes Maggiore,
210 Como and Iseo), Switzerland and France, with a number of clearly underestimated cases
211 due to the common sub-clinical course of the disease (Dupuoy-Camet and Peduzzi,
212 2004; Vaiani et al., 2006; Wicht et al., 2007b).
213 Among the critical aspects responsible for the persistance of diphyllobothriasis in sub-
214 alpine lake areas, the ineffective control of sewage discharge into lakes or through river
215 tributaries plays a key-role.
216 Until the 1950s in Finland, human diphyllobothriasis affected more than 20% of the
217 human population, with a peak of 100% in citizens residing in the eastern region.
218 Through the systematic channeling of urban wastewater and sewage treatment, after 20
219 years these values had dropped to 2-10% (Dick et al., 2001). Currently in Finland as in

220 Sweden, almost 20 cases of human diphyllobothriasis are reported per year (Lavikainen,
221 2010).
222 In Italy the lakes under study still have the same issues observed in the past and the
223 prevalence values of plerocercoids in susceptible fish and urban fecal contamination are
224 still middle to high, as confirmed by the environmental analyses carried out by
225 Legambiente in 2014 (www.legambiente.it), which reported high levels of
226 microbiological water pollution in several sites of these lakes.
227 Despite this evidence, the current situation in Italy seems to have changed with regard
228 to the emergence/riemergence of human diphyllobothriasis in these areas. During this
229 survey only one case from Lake Maggiore and four cases from Lake Iseo were
230 diagnosed, with an unexpected negativity from Lake Como (Bolis, pers.comm.) where
231 previously approximately 10 cases had been diagnosed per year (Terramocci et al.,
232 2001; Vaiani et al., 2006) and despite the high positivity for *D. latum* found in the fish.
233 This change could be due to several factors such as the application of the EC "Hygiene
234 Package" Regulations concerning fish inspection for detecting parasites along the whole
235 chain, up to retail and preventive freezing treatment aimed at killing viable parasites in
236 fishery products intended for raw consumption, the ever-increasing use of specific
237 HACCP programs by fishermen/fish processors and retailers and the enactment of a
238 recent Decree by the Italian Ministry of Health (Decreto Legislativo del 17 luglio 2013)
239 focused on providing consumers with correct information at fishery sales desk ("*In case*
240 *of consumption as raw, marinated or not fully cooked, the product must first be frozen*
241 *for at least 96 hours at -18 °C in a domestic freezer marked with three or more stars*").
242 These preventive measures are particularly important in the peculiar reality of
243 professional freshwater fisheries, which are usually small-scale retailers in which

potentially infected fish follow short supply chains “from capture to plate”, and considering that in Italian sub-alpine areas restaurants propose raw perch (“perch carpaccio”) as a typical local dish, although in the past the traditional local cuisine only included well-cooked lake fish.

In this regard it is important to note that *D. latum* plerocercoid larvae are devitalized rapidly by temperatures of 50 °C for 10’ or > 56 °C for 5’ (Acha and Szyfres, 2003; Peduzzi and Boucher-Rodoni, 2001). They are also easily killed by low temperatures, such as -10 °C for 48 hours (Acha and Szyfres, 2003). Therefore, besides cooking, preventive freezing at -20 °C for 24 hours or at -35 °C for 15 hours, as prescribed for fishery products intended to be administered raw, undercooked, pickled or cold-smoked by the current EU Regulation No. 1276/2011, amending Annex III of Regulation (EC) No 853/2004, is effective in killing *D. latum* plerocercoid larvae.

In conclusion, two main risk factors may be identified for the persistance of diphyllobothriasis due to *D. latum* in the sub-alpine Italian lakes: the ineffective sewage treatment systems that can still be found along the coasts, which lead to the contamination of lake waters by cestode eggs shed by infected humans, and the new eating habit of consuming raw lake fish, in particular perch which have a high prevalence of *D. latum* plerocercoid larvae in the muscles.

In anticipation of solving the first critical point, the implementation of appropriate HACCP programs and the application of regulations from the primary production to the final consumer, together with healthcare education concerning eating habits all play important roles in preventing this fish-borne parasitic zoonosis and minimizing the impact of human diphyllobotriasis in areas where the larval stage of the parasite is present in fish populations.

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390 Figure Legends:

391 Fig 1: map of sampling sites in sub-alpine Italian Lakes.

392 Fig 2: plerocercoid larva of *D. latum* from fillet of perch (*Perca fluviatilis*)

393

394 Table 1. Fish sampled from Maggiore, Como, Iseo and Garda Lakes

395

Fish species	Maggiore Lake	Como Lake	Iseo Lake	Garda Lake	Total
Perca fluviatilis	635	426	458	313	1832
Alosa fallax lacustris	28	0	113	31	172
Coregonus spp.	26	0	51	27	104
Lota lota	0	55	26	11	92
Esox lucius	1	19	7	1	28
Total	690	500	655	383	2228

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397

398 Table 2. Prevalence, intensity and abundance values of *Diphyllobothrium* plerocercoid
399 larvae in the fish species examined
400

Fish species	n examined	n. positive	P (%)	Infection intensity			A
				Min	Max	MI	
Perca fluviatilis	1832	185	10.1	1	4	1.21	0.12
Alosa fallax lacustris	172	0	-	-	-	-	-
Coregonus spp.	104	0	-	-	-	-	-
Lota lota	92	3	3.3	1	3	2	0.07
Esox lucius	28	22	78.6	1	206	24.32	19.11
Total	2228	210	9.4	1	206	3.64	0.34

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403 Table 3. Prevalence with binomial confidence intervals (BCI 95%), intensity and
404 abundance values of plerocercoid larvae in relation to the fish species examined in
405 Maggiore, Como and Iseo Lakes respectively.
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Maggiore Lake							
Fish species	n. examined	n. positive	P % (BCI 95%)	Infection intensity			A
				Min	Max	MI	
P. fluviatilis	635	42	6.6 (4.8-8.8)	1	3	1.05	0.07
A. fallax lacustris	28	0	0 (0-12.3)	0	0	0	0
Coregonus spp.	26	0	0 (0-13.2)	0	0	0	0
E. lucius	1	1	100 (1-2.5)	1	1	1.00	1.00
L. lota	0	0	-	-	-	-	-
Total	690	43	6.2 (4.5-8.3)	1	3	1.05	0.07
Como Lake							
P. fluviatilis	426	108	25.4 (21.3-29.8)	1	3	1.24	0.31
L. lota	55	2	3.6 (0.4-12.5)	1	2	1.50	0.05
E. lucius	19	16	84.2 (60.4-96.6)	2	206	28.25	23.79
Coregonus spp.	0	0	-	-	-	-	-
A. fallax lacustris	0	0	-	-	-	-	-
Total	500	126	25.2 (21.5-29.2)	1	206	4.66	1.11
Iseo Lake							
P. fluviatilis	458	35	7.6 (5.4-10.5)	1	4	1.29	0.10
A. fallax lacustris	113	0	0 (0-3.2)	0	0	-	0
Coregonus spp.	51	0	0 (0-7.0)	0	0	-	0
L. lota	26	1	3.8 (0.1-19.6)	3	3	3.00	0.12
E. lucius	7	5	71.4 (29.0-96.3)	1	35	16.40	11.71
Total	655	41	6.3 (4.5-8.4)	1	35	3.17	0.20

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