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1 **Prevalence of *Diphyllbothrium latum* (Cestoda: Diphyllbothriidae) plerocercoids**
2 **in fish species from four Italian lakes and risk for the consumers**

3
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26 **Summary**

27 In recent years there has been a re-emergence of diphyllbothriasis by
28 *Diphyllbothrium latum* (Cestoda: Diphyllbothriidae) in Italy, France and Switzerland,
29 where in the past this fish-borne zoonosis was widespread and then virtually
30 disappeared. A change in eating habits such as the consumption of raw/undercooked
31 freshwater fish, has led to an increased risk for consumers of ingesting infective larvae
32 of *D. latum*. A survey on the factors responsible for the re-emergence of human
33 diphyllbothriasis in Italy was carried out from March 2013 to December 2014. The
34 aim of this study was to assess the diffusion of *D. latum* plerocercoids in the fish
35 populations of the sub-alpine lakes of Maggiore, Como, Iseo and Garda, updating the
36 scarce historical data and assessing a preliminary “risk level” of the lacustrine
37 environments and fish species under investigation. A total of 2228 fish belonging to 5
38 species, 690 from Lake Maggiore, 500 from Lake Como, 655 from Lake Iseo and 383
39 from Lake Garda Lake were submitted to parasitological examination. The presence of
40 *D. latum* plerocercoid larvae was detected in 6.6%, 25.4% and 7.6% of perch (*Perca*
41 *fluviatilis*) from Lakes Maggiore, Como and Iseo respectively. The parasite was also
42 present in pike (*Esox lucius*) with prevalence values ranging from 71.4 to 84.2% and in
43 3.6-3.8% of burbot (*Lota lota*) from Lakes Iseo and Como. Fish from Lake Garda were
44 negative as well as sampled whitefish (*Coregonus lavaretus*) and shad (*Alosa fallax*
45 *lacustris*). The results of this survey showed a widespread presence of *D. latum*
46 plerocercoid larvae in Maggiore, Como and Iseo fish populations. Urban fecal
47 contamination of water is still a key issue to be resolved, together with the improvement
48 of communication with consumers regarding the best dietary habits and the most
49 effective processes of parasite inactivation, required for the consumption of
50 raw/undercooked fish caught in high-risk areas.

51

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

53

54 **1. Introduction**

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

61 *Diphyllbothrium latum* is the most widespread species and since its discovery back in
62 3600 B.C. (Dommelier-Espejo, 2001) the subalpine area between France, Italy and
63 Switzerland has been the scene of a number of episodes of human infections.

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

74 In recent years, there has been an increasing occurrence of human diphyllbothriasis in
75 the Alpine areas of Switzerland, France and Italy (Dupouy-Camet and Peduzzi, 2004; de
76 Marval et al., 2013; Terramocci et al., 2001; Vaiani et al., 2006; Wicht et al., 2007b).

77 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 diphyllbothriasis 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.

102

103 **1. Materials and Methods**

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

109 The fish species examined were European perch (*Perca fluviatilis*), pike (*Esox lucius*),
110 burbot (*Lota lota*), whitefish (*Coregonus* spp.) and shad (*Alosa fallax lacustris*) (Table
111 1). The sample size has been calculated for perch considering an expected prevalence of
112 5%±2.5% (CI 95%). A sample size of at least 281 fish to be examined for each lake has
113 been considered. The other fish species have been sampled when available.

114 The fish were caught by professional fishermen using gill nets, immediately stored in
115 cold boxes and then transported to the Laboratory of Fish Pathology, Department of
116 Veterinary Medical Science, University of Bologna, Italy. All of the fish were weighed,
117 measured and subjected to parasitological examination according to Roberts et al.
118 (2012). In order to detect the presence of plerocercoids larvae of *D. latum*, the
119 abdominal cavity and fillets were carefully examined by direct observation and/or
120 candling (visual inspection as indicated by EU regulation, in particular Regulation (EC)
121 No 853/2004 and Regulation (EC) No 2074/2005.

122 The plerocercoid larvae were isolated from the host tissue by means of dissecting
123 needles, and were then counted and fixed in 70% ethanol. The morphological
124 identification of the larvae was carried out in accordance with the key provided by
125 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 Advance™ 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 found 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).

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

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

158

159 3. Discussion

160

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

163 Lake Como showed the most worrying prevalence values compared to Lakes Iseo and
164 Maggiore (25.4% vs 7.6% and 6.6%, respectively), meaning that 1 in 4 perches was
165 parasitized by a plerocercoid larval stage. The prevalence values in perch caught in
166 Lakes Como and Iseo substantially confirm previous data from literature (Borroni and
167 Grimaldi, 1973; Gustinelli, 2008; Parona, 1887; Prearo et al., 2013; Scolari, 1955;
168 Wicht et al., 2009), while the fish caught in Lake Garda were always negative as already
169 reported by Parona (1887) and Scolari (1955). On the contrary, Borroni and Grimaldi
170 (1973) found 0.4% of positivity in perch, but the fish examined were bought in the local
171 fish market so it was impossible to trace the origin of the positive fish (Grimaldi pers.
172 comm.).

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

178 In most of the perch examined, the larvae were in the fillets, as typically reported for
179 this fish species. An interesting finding was the unusual presence of plerocercoids in the
180 muscles of the pikes, probably due to a residual migratory attitude of a few larvae. This
181 could represent an additional risk factor for the consumer. However, in Italy the
182 transmission of *D. latum* plerocercoids from pikes to humans is probably due to the
183 accidental ingestion of the parasite during fish evisceration, as this fish species is not
184 generally eaten raw or undercooked. Many fish species have been described as being
185 suitable second intermediate hosts of *D. latum* in various geographical areas. Our data
186 confirm that in continental Europe at least three fish species are suitable hosts for the
187 development/survival of the plerocercoid larvae of *D. latum*: European perch (*Perca*
188 *fluviatilis*), pike (*Esox lucius*) and burbot (*Lota lota*). For this study the fish species
189 were primarily selected according to their importance in the transmission of the
190 plerocercoid larvae of *D. latum*: the European perch has been confirmed as being the
191 most suitable second intermediate host of *D. latum*, and the pike as common paratenic
192 host. According to the results obtained burbot, plays a less important role in transmitting
193 *D. latum* to humans. Whitefish (*Coregonus* spp.) and shad (*Alosa fallax lacustris*) were
194 always negative for plerocercoids thus confirming they are not suitable hosts for *D.*
195 *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 diphyllbothriasis
205 observed in people living along the shores of these lakes. In 1901 Perroncito pointed out
206 the huge diffusion of *Diphyllbothrium 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 persistence of diphyllbothriasis 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 diphyllbothriasis 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 diphyllbothriasis 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 diphyllbothriasis 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

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

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

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

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

268

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272

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389

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
<i>Perca fluviatilis</i>	635	426	458	313	1832
<i>Alosa fallax lacustris</i>	28	0	113	31	172
<i>Coregonus</i> spp.	26	0	51	27	104
<i>Lota lota</i>	0	55	26	11	92
<i>Esox lucius</i>	1	19	7	1	28
Total	690	500	655	383	2228

396

397

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

400

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

401

402

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.

406

Maggiore Lake							
Fish species	n. examined	n. positive	P % (BCI 95%)	Infection intensity			A
				Min	Max	MI	
<i>P. fluviatilis</i>	635	42	6.6 (4.8-8.8)	1	3	1.05	0.07
<i>A. fallax lacustris</i>	28	0	0 (0-12.3)	0	0	0	0
<i>Coregonus</i> spp.	26	0	0 (0-13.2)	0	0	0	0
<i>E. lucius</i>	1	1	100 (1-2.5)	1	1	1.00	1.00
<i>L. lota</i>	0	0	-	-	-	-	-
Total	690	43	6.2 (4.5-8.3)	1	3	1.05	0.07
Como Lake							
<i>P. fluviatilis</i>	426	108	25.4 (21.3-29.8)	1	3	1.24	0.31
<i>L. lota</i>	55	2	3.6 (0.4-12.5)	1	2	1.50	0.05
<i>E. lucius</i>	19	16	84.2 (60.4-96.6)	2	206	28.25	23.79
<i>Coregonus</i> spp.	0	0	-	-	-	-	-
<i>A. fallax lacustris</i>	0	0	-	-	-	-	-
Total	500	126	25.2 (21.5-29.2)	1	206	4.66	1.11
Iseo Lake							
<i>P. fluviatilis</i>	458	35	7.6 (5.4-10.5)	1	4	1.29	0.10
<i>A. fallax lacustris</i>	113	0	0 (0-3.2)	0	0	-	0
<i>Coregonus</i> spp.	51	0	0 (0-7.0)	0	0	-	0
<i>L. lota</i>	26	1	3.8 (0.1-19.6)	3	3	3.00	0.12
<i>E. lucius</i>	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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408