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**Prevalence of *Diphyllbothrium latum* (Cestoda: Diphyllbothriidae) 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 diphyllbothriasis by *Diphyllbothrium latum* (Cestoda: Diphyllbothriidae) 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 diphyllbothriasis 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: *Diphyllbothrium 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. (Dommel-er-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 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.

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).

Prevalence (P), Intensity (mean intensity, MI) and Mean Abundance (A) were calculated according to Bush et al. (1997) by QP 3.0 software version 2.0 (Ròzsa et al., 2000). Exact binomial confidence intervals 95% were calculated using STATA® 11.2. Total DNA was extracted with a commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen) from 20 plerocercoid larvae collected from perch, pike and burbot caught in the 3 sampling sites. The DNA were subjected to multiplex PCR of the cytochrome *c* oxidase 1 (COI mtDNA) following the protocol reported by Wicht et al. (2010), which allows for the simultaneous amplification of *D. latum* (437 bp), *D. pacificum* (318 bp), *D. nihonkaiense* (1232 bp) and *D. dendriticum* (727 bp), the most common species infecting humans. Amplification products were resolved on a 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5X TBE (Molecular Probes - Invitrogen, Carlsbad, CA). The bands were excised and purified by NucleoSpin Gel and PCR Clean-up (Mackerey-Nagel GmbH & Co. KG, Neumann-Neander-Str., Düren Germany) and sequenced with an ABI 3730 DNA analyzer at StarSEQ GmbH (Mainz, Germany). Following assembly with Vector NTI Advance™ 11 (Invitrogen, Carlsbad, CA), the sequences were published in GenBank (KU341698-KU341717).

2. Results

Results of the parasitological analyses in the examined fish species are presented in Table 2. Perch, pike and burbot have been found infected by plerocercoid larvae of *Diphyllbothrium* sp. while whitefish and shad were always negative. Concerning the lacustrine environments, results are displayed in Table 3, with similar positivity in perch from Lakes Maggiore and Iseo and a higher prevalence in Como 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 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 persistance 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

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 persistence of diphyllobotriasis 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.

268

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271 2311360).

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273 **References**

274

275 Acha, P.N., Szyfres, B., 2003. Zoonoses and communicable diseases common to man
276 and animals. Vol. 3. Pan American Health Organization, Washington, DC, 135-139.

277 Andersen, K.I., Gibson, D.I., 1989. A key to three species of larval *Diphyllbothrium*
278 Cobbold, 1858 (Cestoda Pseudophyllidea) occurring in European and North America
279 freshwater fish. Systematic Parasitology 13, 3-9.

280 Bianchini, S., Gustinelli, A., Caffara, M., Prearo, M., Fioravanti, M.L., 2012. Update on
281 diffusion of *Diphyllbothrium latum* plerocercosis in lake fish from Northern Italy
282 and risks for the consumer. Ittiopatologia, 9, 19-32 (in Italian).

283 Borroni, I., Grimaldi, E., 1973. Frequenza dell'infestione muscolare da larve
284 plerocercoidi di *Diphyllbothrium latum* (Cestoda Pseudophyllidea) nel pesce
285 persico (*Perca fluviatilis*) dei laghi italiani (anni 1968-1970). Rivista di
286 Parassitologia 34, 45-54 (in Italian).

287 Borroni, I., Grimaldi, E., 1974. Ecologia dell'infestione da larve plerocercoidi di
288 *Diphyllbothrium latum* (Cestoda Pseudophyllidea) a carico delle specie ittiche del
289 lago Maggiore. Rivista di Parassitologia 35, 129-144 (in Italian).

290 Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets
 291 ecology on its own terms: Margolis et al. revisited. *Journal of Parasitology* 83, 575-
 292 583.

293 Chai, J.Y., Murrel, K.D., Lymbery, A.J., 2005. Fish-borne parasitic zoonoses: status and
 294 issues. *International Journal for Parasitology* 35, 1233-1254.

295 Chiopera, A., Ciammarughi, R., Scalzini, A., Badinelli, M.G., Sueri, L., 1986.
 296 Botriocefalosi umana sul Lago d'Iseo: descrizione di 8 casi emersi in periodo breve
 297 ed in area limitata. *Giornale di Malattie Infettive e Parassitarie*, 38, 268. (in Italian).

298 de Marval, F., Gottstein, B., Webber, M., Wicht, B., 2013. Imported diphyllbothriasis
 299 in Switzerland: molecular methods to define a clinical case of *Diphyllbothrium*
 300 infection as *Diphyllbothrium dendriticum*. *Euro Surveillance*, 18(3), 20355.

301 Dick, T.A., 2008. Diphyllbothriasis: the *Diphyllbothrium latum* human infection
 302 conundrum and reconciliation with a worldwide zoonosis, p. 151–184. In Murrell,
 303 K.D., and B. Fried (eds.), *Food-borne parasitic zoonoses: fish and plant-borne*
 304 *parasites (world class parasites)*, vol. 11. Springer, London, United Kingdom.

305 Dick, T.A., Nelson, P.A., Choudhury, A., 2001. Diphyllbothriasis: update on human
 306 cases, foci, patterns and sources of human infections and future considerations.
 307 *Southeast Asian Journal of Tropical Medicine and Public Health* 32, 59-76.

308 Dommel-Espejo, S., 2001. Contribution à l'étude paléoparasitologique des sites
 309 néolithiques en environnement lacustre dans les domaines jurassien et peri-alpin.
 310 Ph.D. thesis. Université de Reims Champagne-Ardenne, Reims, France, 248 pp.

311 Dupuoy-Camet, J., Peduzzi, R., 2004. Current situation of human diphyllbothriasis in
 312 Europe. *Biologi Italiani* 9, 15-19 (in Italian).

313 EC REGULATION No 853/2004 of the European Parliament and of the Council of 29
 314 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs.

315 EC REGULATION No 1276/2011 of 8 December 2011 amending Annex III to
 316 Regulation (EC) No 853/2004 of the European Parliament and of the Council as
 317 regards the treatment to kill viable parasites in fishery products for human
 318 consumption.

319 EFSA Panel on Biological Hazards (BIOHAZ) 2010. Scientific Opinion on risk
 320 assessment of parasites in fishery products. EFSA Journal, 8, 4, 1543 [91 pp.].

321 Grassi, L., De Carneri, I., 1969. A case of bothriocephaliasis probably contracted on
 322 Lake Como. Rivista di Parassitologia 30, 101-104.

323 Gustinelli, A., 2008. Elminti di interesse zoonosico in specie ittiche dulciacquicole
 324 nazionali. Tesi di Dottorato di ricerca in Epidemiologia e Controllo delle Zoonosi,
 325 XX ciclo, Alma Mater Studiorum Università di Bologna: 151 pp. (in Italian).

326 Kuchta, R., Brabec, J., Kubáčková, P., Scholz, T., 2013. Tapeworm *Diphyllbothrium*
 327 *dendriticum* (Cestoda)—Neglected or Emerging Human Parasite? PLoS Neglected
 328 Tropical Diseases. 7(12), e2535.

329 Lavikainen, A., 2010. Human medical view on zoonotic parasites. Acta Veterinaria
 330 Scandinavica 52(Suppl. 1), S4.

331 Morishita, K., Komiya, Y., Matsubayashi, H., 1973. Progress of medical parasitology in
 332 Japan. Ed. Meguro Parasitological Museum, Tokyo, Japan. 5, 129-144.

333 Pampiglione, S., Di Guardo, G., 1968. Bothriocephaliasis on Lake Maggiore: a propos
 334 of a new human case. Rivista di Parassitologia 29, 191-196. (in Italian)

335 Parona, C., 1887. Intorno la genesi del *Botriocephalus latus* (Bremse) e la sua frequenza
 336 in Lombardia. Archivi delle Scienze Mediche 11, 41-95. (in Italian)

337 Parona, C., 1894. L'elmintologia italiana: da' suoi primi tempi all'anno 1890. Atti della
 338 Regia Università di Genova: 13, 734.

339 Peduzzi R., Boucher-Rodoni, R., 2001. Resurgence of human bothriocephalosis
 340 (*Diphyllbothrium latum*) in the subalpine lake region. Journal of Limnology 60, 41-
 341 44.

342 Perroncito, E., 1901. I parassiti dell'uomo e degli animali utili e le più comuni malattie
 343 da essi prodotte. Biblioteca Medica Italiana, Casa Editrice Vallardi, Milano. 632 pp.

344 Prearo, M., Pavoletti, E., Gustinelli, A., Caffara, M., Righetti, M., Bona, M.C., Scanzio,
 345 T., Ru, G., Fioravanti, M.L., 2013. *Diphyllbothrium latum* in Italy: plerocercoids
 346 larvae distribution in perch (*Perca fluviatilis*) fillets. Italian Journal of Food Safety 2,
 347 3-4.

348 Regulation (EC) No 853/2004 laying down specific hygiene rules for on the hygiene of
 349 foodstuffs.

350 Regulation (EC) No 2074/2005 laying down implementing measures for certain
 351 products under Regulation (EC) No 853/2004.

352 Roberts, R.J., Smail D.A., Munro E.S., 2012. Laboratory methods. In: Fish Pathology.
 353 4th Edition. Roberts, R.J. (ed). Wiley-Blackwell, Oxford, UK: 439-481.

354 Rózsa, L., Reiczigel, J., Majoros, G., 2000. Quantifying parasites in samples of hosts.
 355 Journal of Parasitology, 86 (2), 228-232

356 Scholz, T., Garcia, H.H., Kuchta, R., Wicht, B., 2009. Update on the human broad
 357 tapeworm (genus *Diphyllobothrium*), including clinical relevance. Clinical
 358 Microbiology Reviews, 22, 146-160.

359 Scolari, C., 1955. Ricerche sulla frequenza della plerocercosi da *Diphyllobothrium*
 360 *latum* nei pesci dei laghi dell'Italia settentrionale e sulla incidenza della infestione
 361 nelle diverse specie ittiche recettive. Clinica Veterinaria 78, 210-214.

362 Terramocci, R., Pagani, L., Brunati, P., Gatti, S., Bernuzzi, A.M., Scaglia, M., 2001.
 363 Reappearance of human Diphyllobothriasis in a limited area of Lake Como, Italy.
 364 Infection 29, 93-95.

365 Vaiani, R., Terramocci, R., Crotti, D., Gustinelli, A., Invernizzi, S., M.L. Fioravanti and
 366 S. Pampiglione, 2006. Diphyllobothriasis in Como Lake, Northern Italy: an update.
 367 Parassitologia 48, 297.

368 Veterinary Regulation on Control Measures of Animal Transmissible Diseases -
 369 Regolamento di Polizia Veterinaria, Decreto del Presidente della Repubblica 8
 370 febbraio 1954, n. 320.

371 Wicht, B., de Marval F., Peduzzi, R., 2007a. *Diphyllobothrium nihonkaiense* (Yamane
 372 et al., 1986) in Switzerland: first molecular evidence and case reports. Parasitology
 373 International 56(3), 195-199.

374 Wicht, B., de Marval, F., Gottstein, B., Peduzzi, R., 2008. Imported diphyllobothriasis
 375 in Switzerland: molecular evidence of *Diphyllobothrium dendriticum* (Nitzsch,
 376 1824). Parasitology Research 102, 201-204.

377 Wicht, B., Gustinelli A., Fioravanti, M.L., Invernizzi, S., Peduzzi, R., 2009. Prevalence
 378 of the broad tapeworm *Diphyllobothrium latum* in perch (*Perca fluviatilis*) and

379 analysis of abiotic factors influencing its occurrence in Lake Lario (Como, Italy).
380 Bulletin of the European Association of Fish Pathologists 29, 58-65.

381 Wicht, B., De Ruggeri, N.F., Riccardi, N., Tonolla, M., De Marta, A., Peduzzi, R.,
382 2007b. Monitoring and molecular characterization of *Diphyllbothrium* spp. in the
383 subalpine lake region. 66th Annual Assembly of the Swiss Society of Microbiology,
384 Interlaken, 1-2 March 2007.

385 Wicht, B., Yanagida, T., Scholz, T., Ito, A., Jiménez, J.A., Brabec J., 2010: Multiplex
386 PCR for Differential Identification of Broad Tapeworms (Cestoda:
387 *Diphyllbothrium*) Infecting Humans. Journal of Clinical Microbiology 48(9), 3111-
388 3116.

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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

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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

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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

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402

Table 3. Prevalence with binomial confidence intervals (BCI 95%), intensity and abundance values of plerocercoid larvae in relation to the fish species examined in Maggiore, Como and Iseo Lakes respectively.

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