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Could Fish Feeding Behaviour and Size Explain Prevalence Differences of the Nematode *Eustrongylides excisus* among Species? The Case Study of Lake Garda

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Abstract: The nematode *Eustrongylides excisus* is a parasite of freshwater fish- and fish-eating birds, with known differences on prevalence values among fish species. Thus, the present study aims to explore the hypothesis that the feeding behavior and the size of fish belonging to different trophic levels could explain such differences. For that, 14 sampling sites were selected to perform a fish parasitological survey on Lake Garda (Italy) during spring-summer 2020. Amplification of nuclear ribosomal internal transcribed spacer (ITS) rDNA sequences of nematodes morphologically ascribable to the genus *Eustrongylides* allowed to identify them as *E. excisus*. From the five studied fish species (*Perca fluviatilis*, *Lepomis gibbous*, *Coregonus lavaretus*, *Alosa fallax lacustris* and *Micropterus salmoides*), only three presented the parasite *E. excisus*: *P. fluviatilis*, *L. gibbous* and *M. salmoides*, with significant differences in prevalence values among species ($p = 0.002$). Additionally, there were differences in prevalence values within the same fish species captured from different sampling sites. Findings showed that mainly piscivorous fish were positive for *E. excisus* and how the prevalence was highest in *M. salmoides*. As regard the fish size, a negative correlation between body size and *E. excisus* was found in *P. fluviatilis* due to the feeding habit of juvenile perch which feed mainly zooplankton and benthic invertebrates (i.e., oligochaetes, which are the first intermediate hosts of *E. excisus*). The study findings advance novel knowledge in the field of pathogens of zoonotic importance in the aquatic environment.

Keywords: food–borne zoonoses; *Perca fluviatilis*; *Lepomis gibossus*; *Micropterus salmoides*; trophic level

1. Introduction

Nematodes ascribable to the genus *Eustrongylides* Jagerskiold, 1909 (Family: Dioctophymatidae) are large, red, grossly visible parasites that infect numerous fish species and fish–eating birds [1]. Many species have been described within the genus but only three are considered valid: *Eustrongylides tubifex*, *E. excisus*, and *E. ignotus* [2]. *Eustrongylides* spp. have a heteroxenous life cycle. Adults inhabit the mucosa of the oesophagus, proventriculus or intestine of piscivore birds such as Ciconiiformes, Anseriformes, Gaviiformes, and Pelecaniformes [3]. The eggs are released through the stool into the aquatic environment where oligochaetes (i.e., Tubificidae and Lumbriculidae) are the first intermediate host [2,4,5]. Planktivorous, benthivorous and pelagic fish, as well as amphibians and/or

reptiles are second intermediate hosts. Eustrongylid nematodes have been described in 17 orders of fish worldwide to date [6–10]. Furthermore, predatory fish such as pikeperch (*Sander lucioperca*) and pike (*Esox* spp.), amphibians, and reptiles [2,11] may act as paratenic hosts and humans as accidental hosts [12]. Species of the genus *Eustrongylides* are freshwater fish–borne zoonotic nematodes, of which *E. excisus* is the most relevant species [13].

Eustrongylidosis occurs after the consumption of raw or undercooked fish products [10,14]; however, human infection has been rarely recorded to date [14,15]. The presence of larvae in the peritoneal cavity results in severe abdominal pain within 24 h after eating contaminated fish [16–18]. Eberhard and Ruiz-Tiben [19] described a case of human infection caused by female *Eustrongylides* spp. larva isolated from the skin of the lower limb. Treatment is surgical and entails removal of the larvae. As in other parasitic zoonotic infections such as diphyllobothriasis and opisthorchiasis, the leading cause is the consumption of raw or semi–cooked infected freshwater fish products [20] in certain cultural and socioeconomical conditions [21]. The occurrence of various zoonotic parasites in lacustrine fish species has been recently reported in Italy, mainly involving cestodes (*Dibothriocephalus latus*), trematodes (*Clinostomum complanatum*), and nematodes (*E. excisus*) [14,22,23]. *Eustrongylides excisus* was isolated in several fish species (i.e., *Perca fluviatilis*, *Atherina boyeri*, *Micropterus salmoides*) in central Italy [14,24,25], and in a subalpine lake in northwest Italy (*P. fluviatilis*, *M. salmoides*, *L. gibbosus*) [10]. On this path, it was noted a significant difference in prevalence values between such fish species [10]. Thus, providing original and novel knowledge, the present study aims to explore if feeding behaviour and size could explain the difference in prevalence values among fish species belonging to different trophic levels. The study was focused on Lake Garda, one the largest subalpine lakes in northern Italy [26]. Indeed, subalpine lakes are valuable resources for tourism, agriculture, drinking water, and fisheries. Recreational and commercial fishing contribute to the economy of local markets. Thus, the consumption of undercooked or raw fish poses potential risks for human health.

2. Materials and Methods

2.1. Study Area

Lake Garda is Italy's largest lake (water volume 49 km^3; surface area 368 km^2) (Figure 1) and among the deepest subalpine lakes in the country (maximum depth −350 m) [26]. The lake is divided into two basins separated by an underwater ridge connecting Punta S. Vigilio with the Sirmione Peninsula. The western basin is largest and deepest while the eastern basin (maximum depth −80 m) makes up less than 7% of the lake's overall volume [27]. The main inflow is the Sarca River at the northern end of the lake; other minor tributaries enter at the western and the northern shore. The diversity of habitats allows for various fish communities, including species of conservation interest or relevant for recreational and commercial fishing [28].

2.2. Parasitological Examination

This parasitological survey was carried out during the spring–summer of 2020. With the collaboration of local commercial fishermen, a total of 423 fish of various species were caught at 14 sampling sites (Figure 1). The sites were selected to cover most of the surface area of the lake devoted to commercial and recreational fishing (Figure 1). Fish were morphologically identified to the species level according to Kottelat and Freyhof [29]: *Perca fluviatilis* (n = 212), *Lepomis gibbous* (n = 129), *Coregonus lavaretus* (n = 42), *Alosa fallax lacustris* (n = 20) and *Micropterus salmoides* (n = 20). The lake depth at the sampling sites ranged from −1 to −110 m.

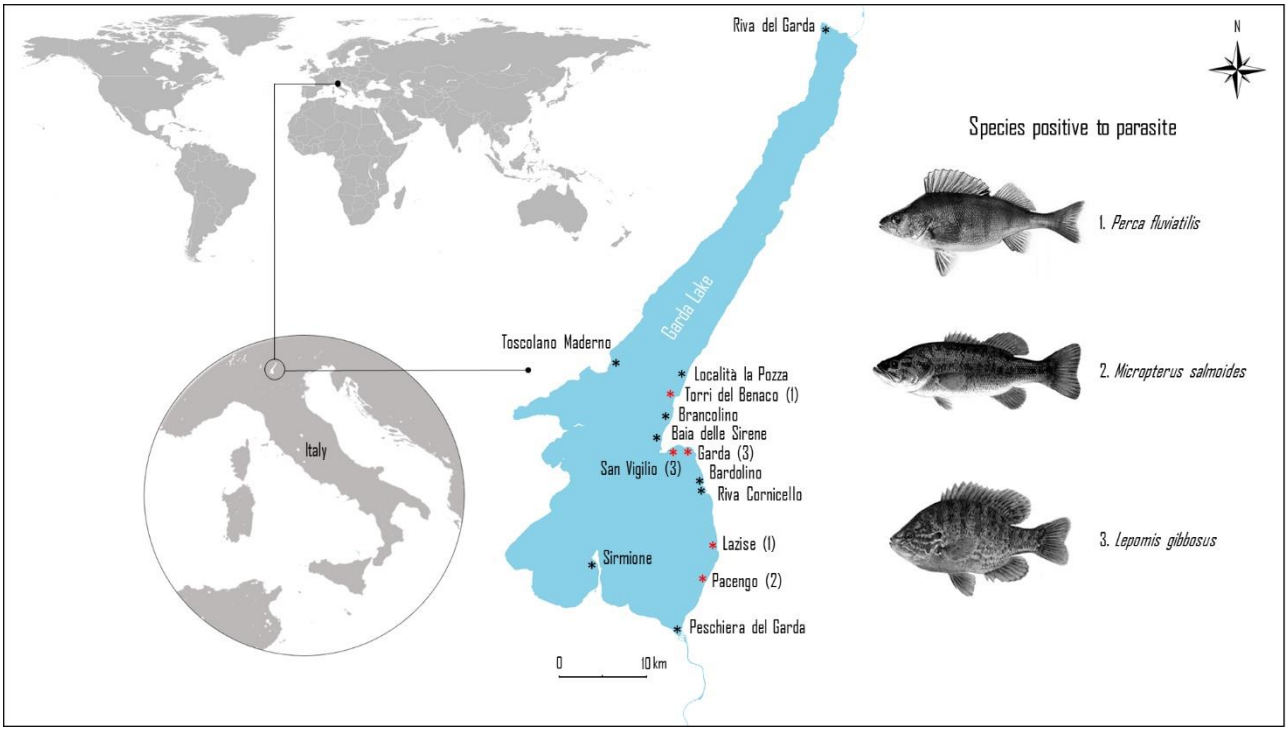

Figure 1. Location of sampling sites (asterisks); where fish species were found positive for *Eustrongylides excisus* are indicated in red asterisks. The numbers in brackets indicate the capture of a single fish species: 1-*Perca fluviatilis*; 2-*Micropterus salmoides*; 3-*Lepomis gibbosus*.

Fish species were caught at a maximum depth of −20 m in the littoral zone. Individuals were collected using mesopelagic gill nets (mesh size 2.5 cm) and kept refrigerated at 4 °C until arrival at the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (Turin, Italy). Biometrical features (total body weight and total length) were recorded for each specimen before anatomopathological examination and sexing. Parasitological examination was performed according to European regulations [30,31]. Briefly, the visceral organs were removed from each individual, placed in Petri dishes containing physiological (salinity 0.9%) solution for visual inspection, and then digested using an enzymatic digestion method based on the Codex Alimentarius Commission [32] and EU Regulation (EC) No. 2075/2005 [30]. The skeletal musculature was sliced (2–3 mm thick), visually observed and inspected by transillumination (UVP white light transilluminators, TW–43, Analytik Jena, Jena, Germany) to detect encysted parasites. The location of each larva was recorded by body quadrant: anterior ventral (AV), anterior dorsal (AD), posterior ventral (VP), posterior dorsal (DP), and visceral cavity (VC). Nematodes were isolated from host tissues with pointed light metal forceps and a fine needle, rinsed in deionized water, and fixed in 70% ethanol. Once fixed, the head and the tail of each larva were removed for morphological examination in light microscopy [33] and the central part of the body devoid of taxonomic features was used for molecular analysis. For observation in scanning electron microscopy (SEM), fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter coated with gold-palladium and observed using a Phenom XL G2 Desktop SEM (Thermo Fisher Scientific) operating at 5 kV.

2.3. Molecular Analysis

Molecular analyses were carried out following Mazzone et al. [34]. Briefly, genomic DNA was extracted from four nematodes by means of a PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Amplification of the complete internal transcribed spacer (ITS) rDNA region was performed with primers 81_f-GTAACAAGGTTTCCGTAGGTGAA and ITS2.S_r–CCTGGTTAGTTTCTTTTCCTCCGC [35,36]. For the amplification, molecular grade water

was used as a negative control, while a sequenced sample of *Eustrongylides excisus* from a previous study [34] was used as positive control. The amplified products were electrophoresed on 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5× TBE. The amplicons were purified with Nucleo–Spin Gel and PCR Cleanup (Mackerey–Nagel, Düren, Germany) and sequenced on an ABI 3730 DNA analyser (StarSEQ, Mainz Germany).

The trace files were assembled with ContigExpress (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA, USA) and the consensus sequences were compared with previously published data using BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 12 December 2021). The sequences generated in this study were deposited in GenBank under accession numbers MZ648893-MZ648894.

2.4. Statistical Analysis

Normality and homoscedasticity of data were assessed through the Shapiro–Wilk and Levene tests, respectively. Prevalence, mean intensity, and mean abundance of infestation were calculated for each species according to Bush et al. [37]. Differences in the prevalence of infestation between the fish species were tested using the chi-square test and the 95% confidence intervals (95% CI) were calculated. Since the null hypothesis for normal distribution could not be rejected, the non–parametric Kruskal–Wallis or Mann–Whitney U tests were used to reveal differences in biometrical features between the fish species per sampling site (only those that were tested positive for *E. excisus*) and differences in the site of larvae infestation in musculature quadrants [anterior ventral (AV), anterior dorsal (AD), posterior ventral (PV), posterior dorsal (PD)] and visceral cavity (VC). The Conover–Iman post–hoc test was used for multiple comparisons since the number of individuals for certain fish species was <20 (Table 1). Spearman's rank correlation coefficient (ρS) was used to test for correlations between total body length and infection with nematode larvae. Significance was set at 0.05%. All basic statistics and analyses were carried out using R software (v. 4.0.5; R Core Team, 2021).

Table 1. Biometrical features (weight and total length; mean ± standard deviation), sex, and number (N) of fish species from sampling sites in Lake Garda. ND = sex not determined. Lowercase letters denote differences revealed by the Conover–Iman post–hoc test.

Sampling Site	Species	Weight (g)	Total Length (cm)	N	Male	Female	ND
Peschiera del Garda	*Alosa fallax*	57.1 ± 13.0	19.5 ± 1.9	20	45	55	—
Lazise	*Coregonus lavaretus*	120.2 ± 28.0	23.9 ± 2.1	6	50	50	—
Pacengo		91.8 ± 80.4	20.1 ± 7.1	5	50	50	—
Peschiera del Garda		99.8 ± 9.1	23.0 ± 0.6	5	60	40	—
Riva del Garda		321.5 ± 53.8	31.8 ± 3.2	5	0	100	—
Sirmione		115.21 ± 20.5	23.4 ± 1.0	10	50	50	—
Toscolano Maderno		277.2 ± 50.5	30.1 ± 1.9	11	45.5	54.5	—
Bardolino	*Lepomis gibbosus*	50.6 ± 41.9 ^{b}	12.2 ± 3.0 ^{b}	30	26.7	50.0	23.3
Garda		26.0 ± 12.4 ^{b}	11.0 ± 1.4 ^{b}	27	34.6	65.4	—
San Vigilio		86.4 ± 23.2 ^{b}	16.5 ± 2.0 ^{a}	24	54.2	45.8	—
Toscolano Maderno		62.3 ± 38.9 ^{b}	13.29 ± 5.8 ^{b}	48	37.5	62.5	—

Table 1. *Cont.*

Sampling Site	Species	Weight (g)	Total Length (cm)	N	Male	Female	ND
Pacengo	*Micropterus salmoides*	93.0 ± 48.8 [a]	33.5±29.1 [a]	10	40	60	—
Torri del Benaco		1168.7 ± 571.0 [b]	40.3 ± 3.2 [b]	10	66.7	33.3	—
Baia delle Sirene	*Perca fluviatilis*	120.4 ± 33.4 [b]	20.2 ± 1.8 [b]	5	0	100	—
Brancolino		120.5 ± 27.3 [b]	21.0 ± 1.3 [b]	14	21.4	78.6	—
Garda		99.36 ± 20.6 [b]	20.41 ± 1.5 [b]	11	18.2	81.8	—
Localita la Pozza		125.3 ± 31.6 [b]	20.8 ± 1.9 [b]	4	25	65	—
Lazise		94.7±24.6 [a]	18.7 ± 1.9 [a]	30	3.3	96.7	—
Riva Cornicello		111.5±26.8 [b]	20.6 ± 1.6 [b]	11	0	100	—
San Vigilio		122.4 ± 42.4 [b]	21.0 ± 2.0 [b]	12	16.7	83.3	—
Torri del Benaco		98.6 ± 32.6 [b]	20.9 ± 2.0 [b]	65	16.9	83.1	—
Toscolano Maderno		100.6 ± 33.8 [b]	20 5 ± 2.1 [b]	60	45	65	—

3. Results

Fishes resulted positive for the presence of *E. excisus* larvae were 13 out of 423 (3.07%) and all isolated parasite larvae were morphologically referred to the genus *Eustrongylides* spp. (Figures 2 and 3). Among sampled fish species, only in *Perca fluviatilis, Lepomis gibbosus* and *Micropterus salmoides,* (Figure 3) larval stage of *Eustrongylides* were isolated. To note, no visible lesions in the external or internal organs were observed. All other fish species tested negative for nematode larvae. The prevalence of *Eustrongylides* spp. was: 12.5% (95% CI, 4.3–31%) and 3.70% (95% CI, 0.19–18.28%) in *L. gibbosus* from San Virgilio and Garda, respectively; 5.55% (95% CI, 0.99–18.14%) and 1.54% (95% CI, 0.08–8.21%) in *P. fluviatilis* from Lazise and Torri del Benaco, respectively; and 60% (95% CI, 31.27–83.18%) in *M. salmoides* from Pacengo. There were significant differences in nematode prevalence between fish species (chi-square, $p = 0.002$). There were significant differences in total length (Kruskal–Wallis, $p = 0.005$) and weight (Kruskal–Wallis, $p = 0.004$) of *P. fluviatilis* between sampling sites, with significant differences between Lazise and the other sites (Conover–Iman post-hoc test, $p < 0.05$ for all comparisons) (Table 1). A significant difference in total length ($p = 0.001$) and weight ($p = 0.002$) was noted for *L. gibbosus* between the sampling sites, with a marked difference between San Virgilio and the other sites (Conover–Iman post-hoc test, $p < 0.05$ for all comparisons) (Table 1). Total length and weight significantly differed in *M. salmoides* between Pacengo and Torri del Benaco (Mann–Whitney U-test; $p = 0.001$ and $p = 0.003$ for total length and weight, respectively). Spearman's rank correlation showed a significant negative correlation between total body length and infection with *Eustrongylides* spp. in *P. fluviatilis* (ρs −0.140; $p = 0.02$). Table 2 presents the number, location (AV, AD, PV, PD muscular quadrants and VC) of nematode larvae, mean intensity, and mean abundance of infestation. There was a significant difference in larvae localization (AV = 8; PV = 1; AD = 2; PD = 2; VC = 2) (Kruskal–Wallis test, $p = 0.008$), with the highest number recorded in the AV muscle quadrant (Conover–Iman post-hoc test, $p < 0.05$ for all comparisons) (Table 2). The mean intensity (MI) of infestation ranged from 1 in *P. fluviatilis* and *L. gibbosus* to 1.33 in *M. salmoides*; the mean abundance (MA) ranged from 0.015 to 0.05 in *P. fluviatilis* and from 0.03 to 0.12 in *L. gibbosus* and was 0.8 in *M. salmoides* from Pacengo.

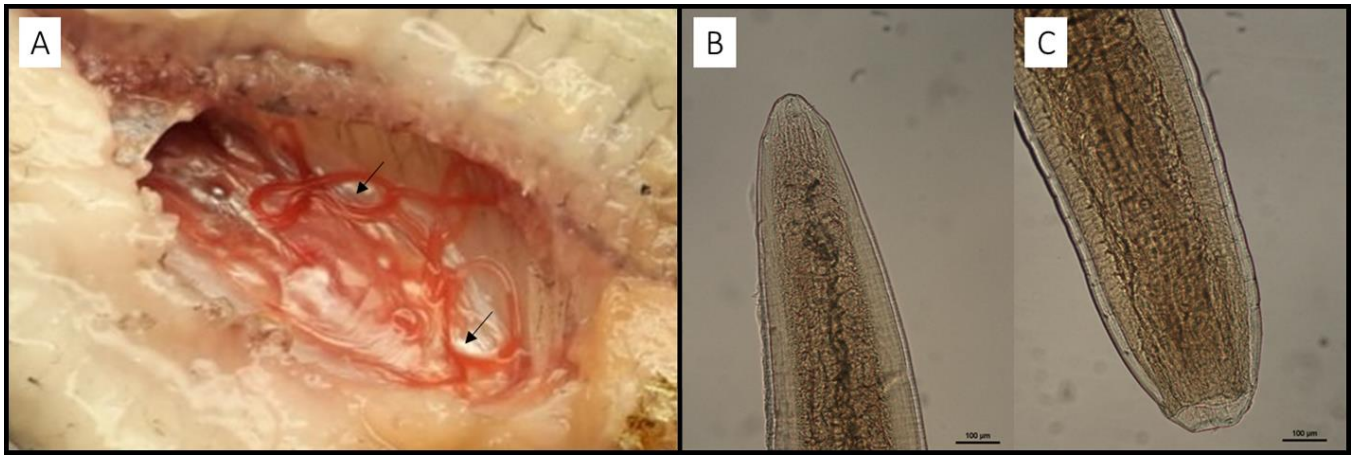

Figure 2. (**A**) Macroscopic examination of the sampled fish, and parasites located in the swim bladder (black arrows). *Eustrongylides excisus* larva: anterior end (**B**), and tail (**C**).

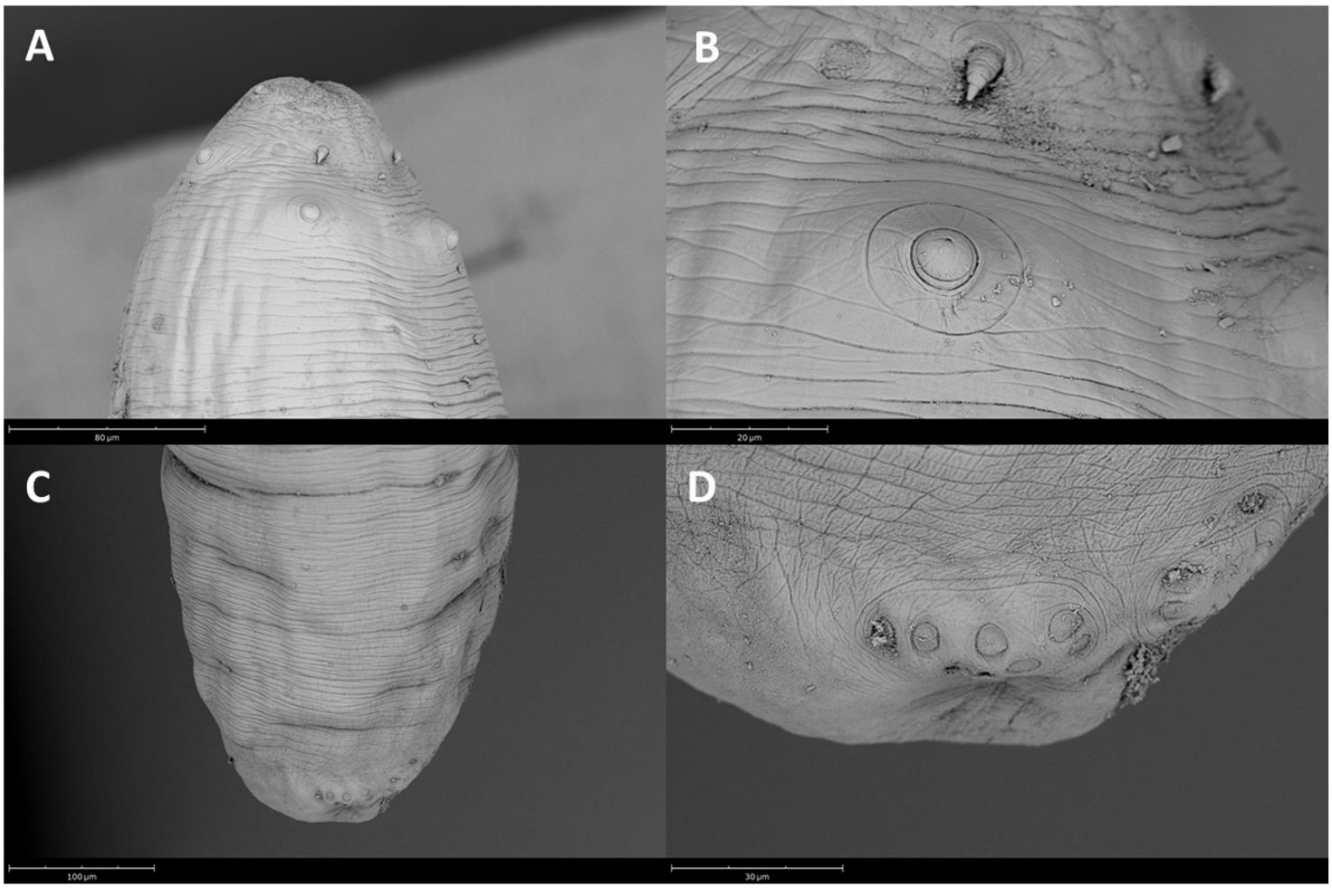

Figure 3. SEM micrographs of *Eustrongylides excisus* larva. (**A**): anterior end; (**B**): detail of cephalic papillae; (**C**): tail; (**D**): detail of caudal papillae.

Table 2. Number of fish analysed (N) and prevalence of *Eustrongylides excisus* in freshwater fish species from Lake Garda. The number and the location [lowercase letters: anterior ventral (AV), anterior dorsal (AD), posterior ventral (VP), posterior dorsal (DP), visceral cavity (VC)] of nematode larvae, and the mean intensity (MI) and mean abundance (MA) for each fish species are reported.

Sampling Site	Fish Species	N	Positive	P (%; 95% CI)	N of larvae	MI	MA
Lazise	*Perca fluviatilis*	36	2	5.55 (0.99–18.14)	2^{AV}	1	0.05
Torri del Benaco	*Perca fluviatilis*	65	1	1.54 (0.08–8.21)	1^{AV}	1	0.015
Pacengo	*Micropterus salmoides*	10	6	60 (31.27–83.18)	3^{AV};2^{AD};1^{PV};2^{VC}	1.33	0.8
Garda	*Lepomis gibbosus*	27	1	3.7 (0.19–18.28)	1^{AV}	1	0.03
San Vigilio	*L. gibbosus*	24	3	12.5 (4.3–31)	1^{AN}; 2^{PD}	1	0.12

Amplification of the ITS rDNA sequences of nematodes morphologically ascribable to the genus *Eustrongylides* spp. showed they were identical to each other. BLAST analysis gave 100% identity with the sequence of *E. excisus* deposited by Mazzone et al. [34] and 99.73–99.74% identity with those of Pekmezci et al. [1].

4. Discussion

Parasites are an integral but frequently neglected part of ecosystems, where they cause either direct (e.g., mechanical destruction of cells) or indirect damage (e.g., withdrawal of nutrients and intoxication) to the host [38]. Fishery products are marketed as high-quality food items, making the question about the potential risk of infection with fish borne zoonotic parasites in humans highly relevant for public health. Recent studies have focused on the incidence and geographic distribution of *Eustrongylides* spp. in Italy [10,14,24,25]. The expansion of the geographical range of *E. excisus* may be linked to the recent rise in number and area of the cormorant population in northern Italy, where it is concentrated around large lakes and other pre-alpine water basins [10,39]. According to Atkinson et al. [40] the increase in ichthyophagous bird populations (final hosts), plays a critical role in the incidence of *Eustrongylides* spp. in fish. The prevalence we recorded in *P. fluviatilis* (1.54%) was lower than the 10%, 6.84% and 6% reported by [10,24,25], respectively. The prevalence value of 60% in *M. salmoides* reported here was higher than the 1.89% and the 10% reported by Branciari et al. [24] and Menconi et al. [10], respectively. In a recent paper, Menconi et al. [10] noted that the prevalence of *L. gibbosus* (18.3%) was far higher than what we recorded in this study. The difference could be related to the remote locations of the lakes they sampled, which were areas probably more favourable for nesting and habitation of fish-eating birds that are the final hosts for *E. excisus* [41]. Other environmental factors that may also contribute to the occurrence of *E. excisus* in fish species in Lake Garda include the biological characteristics and the abundance of intermediate and final hosts. For example, environmental conditions favourable for the growth of oligochaete populations play a pivotal role in sites with high prevalence of *E. excisus* in fish [9]. Additionally, as expected, the feeding behaviour of fish may explain the range of infected species: predatory fish may consume several infected preys and thus amplify the intensity of parasite infection [9]. The relationship between eutrophication and *Eustrongylides* spp. prevalence is another a key element to understand the epizootiology of this parasite. We found that mainly piscivorous fish were positive for *E. excisus* and that prevalence was highest in *M. salmoides*. Fish belonging to the centrarchidae family are the most important paratenic hosts [9]; however, since the frequency of fish predation by *L. gibbosus* is generally low, it probably acquires *Eustrongylides* spp. by feeding on infected oligochaetes [4]. As regard the fish size, the negative correlation between body size and *Eustrongylides* spp. infection in *P. fluviatilis* may be due to the feeding habit of juvenile perch [10], which feed mainly on zooplankton and benthic invertebrates (i.e., oligochaetes), while adults feed on macroinvertebrates and other fish [42–44]. Based on our data we could hypothesize that the high prevalence in young perch possibly results from feeding on infected oligochaetes.

The difference in parasite localization between muscular quadrants (AV = 8; PV = 1; AD = 2; PD = 2) and visceral cavity (VC = 2) could be due to larval migration after infestation of the fish host. Larvae acquisition occurs through the ingestion of infected prey, therefore their localization in the anterior ventral quadrant may be favoured. The European perch is an economic resource for local fishermen, and it is widely used in traditional cuisine. Within the present study, we particularly focused on this fish species as it is a suitable host for *E. excisus*.

Eustrongylides excisus infection in this commercially relevant fish may adversely affect consumer perception and pose a health risk. Other fish species (e.g., *Coregonus lavaretus*, and *Alosa fallax lacustris*) important for local commercial fisheries, they tested negative possibly due to the fact that they feed on planktonic crustaceans.

Changes in eating habits, increased global fish trading, and advances in transportation technologies have brought about considerable changes in the epidemiology of fish–borne parasitic zoonoses [15]. In response to the rising popularity of marinated, raw, or semi–cooked and ethnic fish dishes, health authorities have implemented measures to manage the risk of food–borne illness [30,31,45,46]. In addition, the Italian Ministry of Health [47] has introduced the concept of mandatory consumer information by fish sellers. Fish shops in Italy must display a sign with the following recommendation: "*Products intended for raw consumption, marinated or not fully cooked, should be frozen for at least 96 h at −18 °C in a domestic freezer marked with three or more stars*" [47]. These measures regard certain zoonotic parasites. Consumers can protect against zoonotic helminth infection by simply freezing fish that will be consumed raw or cooking.

5. Conclusions

The increased prevalence of *Eustrongylides* spp. in Italian lacustrine fish suggests its recent spread. Results from this study showed how *Eustrongylides excisus* prevalence was highest in piscivorous fish as *Micropterus salmoides*, suggesting that feeding behaviour could explain the difference in prevalence values among fish species belonging to different trophic levels. Moreover, the negative correlation found between body size and *E. excisus* infection in *Perca fluviatilis* clarifies the higher prevalence found in juvenile perch which feed mainly on zooplankton and benthic invertebrates as oligochaetes (the first intermediate hosts of *E. excisus*). Finally, this study integrates current data about *E. excisus* geographical distribution and fish host range in lacustrine environments. However, further studies are needed since numerous aspects of the biology, epidemiology, and control of *Eustrongylides* species are still scarce to date.

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