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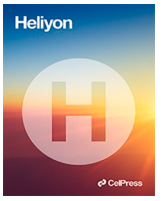

Research article

Mapping the occurrence of *Eustrongylides* spp. in fish species caught from six lakes in Central Italy (Tuscany and Latium regions): Implications for local fishery supply chains

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ABSTRACT

In the present study, in order to expand the knowledge on the distribution of parasites of the genus *Eustrongylides* in never investigated Italian lacustrine areas of Tuscany and Latium, fish specimens from four Tuscan lakes (Bilancino, Chiusi, Montedoglio and San Cipriano) and two Latium lakes (Bolsena and Bracciano) were collected and analyzed. The parasitological analysis, consisting of a visual inspection followed by a chloro-peptic digestion, was performed on 1650 specimens belonging to 17 species: European perch (*Perca fluviatilis*), Pumpkinseed (*Lepomis gibbosus*), Pike-perch (*Sander lucioperca*), Largemouth black bass (*Micropterus salmoides*), Pike (*Esox lucius*), Big-scale sand smelt (*Atherina boyeri*), Tench (*Tinca*), European Whitefish (*Coregonus lavaretus*), Channel catfish (*Ictalurus punctatus*), Chub (*Squalius cephalus*), Rudd (*Scardinius erythrophthalmus*), Common bleak (*Alburnus alborella*), South European roach (*Sarmarutilus rubilio*), South European nase (*Protochondrostoma genei*), Italian riffle dace (*Telestes muticellus*), Goldfish (*Carassius auratus*), and Freshwater bream (*Abramis brama*). *Eustrongylides* spp. larvae were only found in one specimen of European perch caught in Bracciano Lake and purchased from a local fishmonger (P = 3.3%; MI = 1; MA = 0.03). The only isolated *Eustrongylides* spp. larva was submitted to molecular analysis of the ITS gene region and identified as *E. excisus*. This finding adds Bracciano Lake to the list of the several Italian lakes in which nematodes belonging to this genus have been reported. Even if the observed prevalence is currently low, the presence of the parasite in this local freshwater fish supply chain requires FBOs to adopt risk management measures to prevent contaminated products from reaching final consumer. Moreover, future investigations will show if the parasite expands in these areas.

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1. Introduction

Parasites belonging to the genus *Eustrongylides* spp. have an indirect life cycle involving piscivorous birds as definitive hosts, oligochaetes (Anellida phylum) as first intermediate hosts, and benthonic fish as second intermediate hosts [1–3]. Predatory fish species act as paratenic hosts [1,4]. Therefore, *Eustrongylides* spp. larvae can be found both in the viscera and in the edible part (muscle) of a wide variety of freshwater fish species [5–8].

To date, larval nematodes of the genus *Eustrongylides* have been identified as the causative agent in seven cases of human infection (see details in Table 1 in Guardone et al. [9]). Such infections were linked, when described, with the consumption of live minnows or domestically prepared sushi and sashimi [10–13]. According to the WoRMS organization website (World Register of Marine Species, WoRMS) three out of the four known species of *Eustrongylides* spp. (*E. excisus*, *E. mergorum* and *E. tubifex*) are distributed in Europe, including Italy [4,14]. However, no case of human infection has so far been described in this country, nor in Europe [4,9]. The absence of reports of human infection does not necessarily reflect the absence of human cases, and eustrongylidosis could be underdiagnosed, similarly to other nematode-borne infections [15]. This underestimation could be attributed to the fact that often medical doctors do not have full knowledge of nematode-borne human parasitoses and the associated clinical symptoms [16]. The genus *Eustrongylides* (Jägerskiöld, 1909) (Dioctophymatidae family) has a cosmopolitan distribution and it has been reported in fish species in North and South America, Asia, and also in Europe [4,17–20]. In regard to Italy, since the first report of the genus *Eustrongylides* in freshwater fishes caught in Trasimeno Lake (Umbria) in 2014 [21], the distribution of this nematode in national freshwater basins has expanded [8,22]. Indeed, its presence was reported in various fish species and ichthyophagus birds from the Trasimeno Lake (Umbria) [18, 23–25], in fish species from Garda Lake [6,26], Annone Lake [22] (Lombardy) and informally in two other subalpine lakes (Lakes Ceresio and Montorfano) [14]. This parasite was also reported in fish species caught in the Massaciuccoli Lake (Tuscany) [8,9]. More specifically, only the occurrence of the species *E. excisus* was effectively documented in Italy [8,9,18,25,26]. The parasite distribution in Italian lakes is fully detailed in Table 1 in [14]. This apparent spreading may be linked to the increase of wintering and nesting sites of one of its main definitive hosts, the Great cormorant (*Phalacrocorax carbo*) (Linnaeus, 1758) [22,27]. Other phenomena such as eutrophication and the rise of water temperatures, favoring the increase in the population of first intermediate hosts, may contribute to parasite transmission [14]. Moreover, among freshwaters ecosystems and biota (fish and vegetation) a strong quantitative and qualitative interdependence exists, and the diversity and development of the fish population is also affected by anthropogenic activities through the alteration of water quality [28].

Although freshwater fishery products represent only about 5% of the European fish production [29], their commercial interest can be locally relevant [9,14]. In freshwater basins where recreational or professional fishing is practiced, the presence of zoonotic parasites such as *E. excisus* implies possible public health issues if fishery products are intended for human consumption [8,9]. To manage such parasitic risk, Regulation (EC) No 853/2004 [30] prescribes several measures, including immediate evisceration, visual inspection, and thermic treatments (cooking at 60 °C at core for 10 min or freezing at −20 °C at core for 24 h). These legal requirements had been developed and, so far, mainly applied to manage the risk related to the presence of anisakid nematodes in saltwater fishery products [31,32]. Nevertheless, they must also be applied to manage the risk related to other nematodes in fishery products, such as those belonging to the genus *Eustrongylides*, although no specific studies have yet been conducted in this regard [8].

Beyond public health issues related to viable larvae, the presence of *Eustrongylides* spp. larvae can also cause the consumer's repugnance and rejection, even if the parasites are dead [9,14,25]. Indeed, because of their morphological characteristics (bright

Table 1
Sampling results: number of specimens caught for each fish species in each lake.

Family	Species	Bilancino Lake	Chiusi Lake	Montedoglio Lake	San Cipriano Lake	Bolsena Lake	Bracciano Lake	Total
Percidae	European perch (*P. fluviatilis*)	19	–	–	–	30	30	79
	Pumpkinseed (L. *gibbosus*)	92	–	–	–	–	–	92
	Pike-perch (*S. lucioperca*)	11	–	–	–	–	–	11
Centrarchidae	Largemouth black bass (*M. salmoides*)	6	–	–	–	24	30	60
Esocidae	Pike (*E. lucius*)	–	–	–	–	1	–	1
Atherinidae	Big-scale sand smelt (*A. boyeri*)	–	–	–	–	270	154	424
Tincidae	Tench (*T. tinca*)	–	–	–	–	8	28	36
Salmonidae	European Whitefish (*C. lavaretus*)	–	–	–	–	2	30	32
Ictaluridae	Channel catfish (*I. punctatus*)	20	–	–	–	–	–	20
Leuciscidae	Chub (*S. cephalus*)	52	–	–	11	–	–	63
	Rudd (*S. erythrophthalmus*)	7	116	–	–	–	34	157
	Common bleak (*A. alborella*)	32	–	572	–	–	–	604
	South European roach (*S. rubilio*)	26	–	–	–	–	–	26
	South European nase (*P. genei*)	20	–	–	–	–	–	20
	Italian riffle dace (*T. muticellus*)	11	–	–	–	–	–	11
Cyprinidae	Goldfish (*C. auratus*)	–	4	–	1	–	–	5
	Freshwater bream (*A. brama*)	–	–	–	9	–	–	9
Total:		**296**	**120**	**572**	**21**	**335**	**306**	**1650**

pink-red color) and considerable size (3–5,5 cm in length and 0,5-1 mm in diameter), larvae belonging to the genus *Eustrongylides* can be considered as "*visible parasites*" according to Regulation (EC) No 2074/2005 [33]. Products that are obviously contaminated with visible parasites are "*unfit for human consumption*" and therefore "*at risk*", so they cannot be put on the market according to Regulation (EC) No 178/2002 [34]. Their presence can therefore negatively affect small-scale fishery economies due to the loss of marketability of heavily contaminated fishery products [25].

This study represents the most extensive survey so far performed across Italian lakes on *Eustrongylides* spp. in fish species, and it aimed to understand if the related parasitological risk is present in six lakes previously not investigated, four in Tuscany (Bilancino, Chiusi, Montedoglio and San Cipriano) and two in Latium Regions (Bolsena and Bracciano). The study also aimed to assess any potential negative impact on the local supply chains and to provide procedures to manage the risk associated to *Eustrongylides* sp. in freshwater fishery products.

2. Materials and methods

2.1. Fish sampling

Sampling was conducted between April 2022 and May 2023 in six Italian lakes: Bilancino (Province of Florence), Chiusi (Province of Siena), San Cipriano and Montedoglio (Province of Arezzo), Bolsena (Province of Rome), Bracciano (Province of Viterbo). The Provinces of Florence, Siena and Arezzo belong to Tuscany Region while Rome and Viterbo Provinces belong to Latium Region (Fig. 1). The specimens were mainly provided by local fishermen's cooperatives, and only in the case of Bracciano Lake by a fishmonger. All the fishes were already dead when received for the analysis. The sampling was conducted trying to include the largest number of species reported as resident in each lake (Tuscan Lakes) and, in case of the presence of a local supply chain (Latium Lakes), it was mainly focused on species of commercial interest. Once caught, the specimens were immediately processed or stored in a freezer at −20 °C until analysis. Before the parasitological analysis, each specimen was associated with an alphanumerical code, photographed, measured, and weighed individually (big specimens) or as a pool (small specimens).

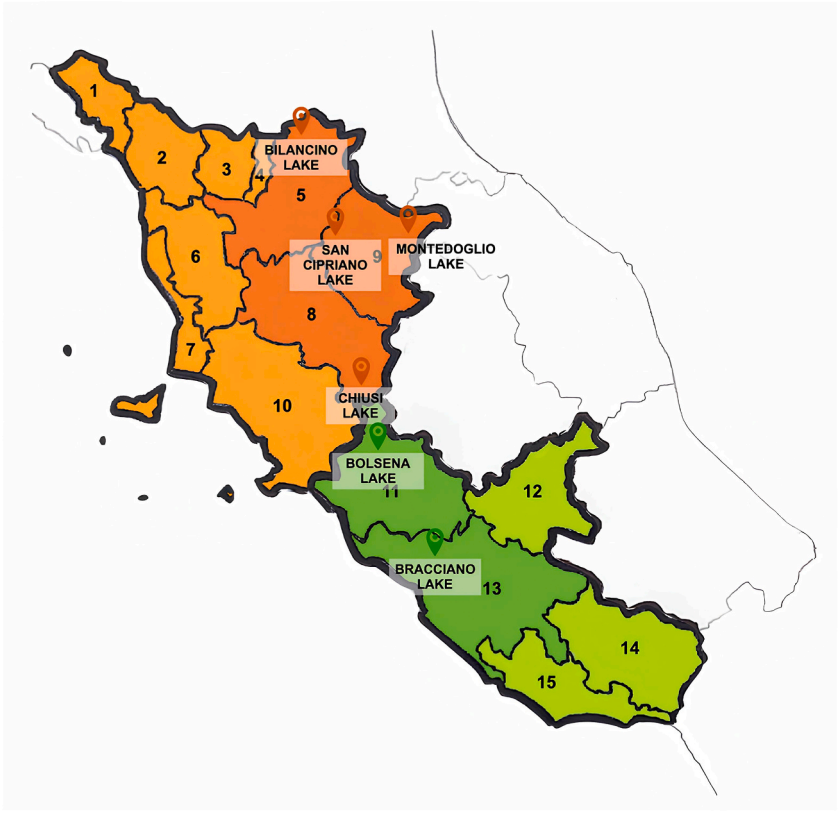

Fig. 1. Sample collection sites. Tuscany: Province of Florence (5), Province of Siena (8), Province of Arezzo (9). Latium: Province of Rome (11), Province of Viterbo (13).

2.2. Parasitological analysis

Specimens were analyzed individually (big specimens) or as a pool (small specimens) for the detection of visible parasites according to Castiglione et al. [8]. Specifically, the parasitological analysis consisted of two steps: visual inspection and chloropeptic digestion.

2.2.1. Visual inspection

All sampled specimens underwent visual inspection of the external surface, viscera and coelomic cavity. Visual inspection was performed in good lighting conditions.

2.2.2. Artificial digestion

An artificial peptic digestion was carried out differently depending on whether the specimen was small or large: for large specimens, viscera and belly flaps were digested separately, while the digestion of small ones was performed on the whole specimen, gathered in pools of ~100 g with other specimens belonging to the same species. Digestion performed in 1 L of water to which 50 ml of 10% HCl and 10 g of PLYtricons® pepsin were added.

2.2.3. Parasite morphological and molecular identification

All the parasites found were collected, counted, and observed microscopically (Nikon Eclipse E 200). Their morphological features, focusing in particular on the cephalic and caudal end, were then compared with the available description of *Eustrongylides* spp. larval stages [1,18,35]. Parasites were stored in 70% alcohol for subsequent molecular identification conducted as described in Castiglione et al. [8].

2.3. Statistical analysis

The prevalence (P) (and 95% confidence intervals – CI), mean abundance (MA) and mean intensity (MI) were calculated according to Bush et al. [36].

3. Results and discussion

3.1. Fish sampling

Overall, 1650 freshwater fish specimens, belonging to 17 species and 9 families were collected in this study. Of these, 1009 specimens were caught in Tuscan lakes (Bilancino, Chiusi, Montedoglio, San Cipriano), while 641 were caught in Latium lakes (Bolsena, Bracciano) (Table 1). As regards Tuscany, 296 specimens belonging to 11 species. *Perca fluviatilis* (Linnaeus, 1758), *Lepomis gibbosus* (Linnaeus, 1758), *Sander lucioperca* (Linnaeus, 1758), *Micropterus salmoides* (Lacepède, 1802), *Ictalurus punctatus* (Rafinesque 1818), *Squalius cephalus* (Linnaeus, 1758), *Scardinius erythrophthalmus* (Linnaeus, 1758), *Alburnus alborella* (Bonaparte, 1841), *Sarmarutilus rubilio* (Bonaparte, 1837), *Protochondrostoma genei* (Bonaparte, 1839), and *Telestes muticellus* (Bonaparte, 1837)) were collected from Bilancino Lake, 120 specimens belonging to two species (*Scardinius erythrophthalmus* and *Carassius auratus* (Linnaeus, 1758)) were collected from Chiusi Lake, 572 specimens belonging to one species (*Alburnus alborella*) were collected from Montedoglio Lake, and 21 specimens belonging to three species (*Squalius cephalus, Carassius auratus, Abramis brama* (Linnaeus, 1758)) were collected from San Cipriano Lake. As for the Latium lakes, 335 specimens belonging to six species (*Perca fluviatilis, Micropterus salmoides, Esox lucius* (Linnaeus, 1758), *Atherina boyeri* (Risso, 1810), *Tinca* (Linnaeus, 1758), *Coregonus lavaretus* (Linnaeus, 1758)) were collected in Bolsena Lake, while for Bracciano Lake, 306 specimens belonging to six species (*Perca fluviatilis, Micropterus salmoides, Atherina boyeri, Tinca, Coregonus lavaretus*, *Scardinius erythrophthalmus*) were purchased from a local fishmonger selling locally sourced fishery products.

No recent publications describing the ichthyofauna of the lakes investigated in this study are available. Nevertheless, the sampling activity was effective in collecting most of the species inhabiting them. In fact, by consulting the websites of the fishermen's federation and association operating in Tuscany and Latium Region respectively, it was possible to verify that the main target species of sport fishing were sampled (https://www.fipsas.it; https://flaglagodibolsena.it). In addition, by consulting online fishmongers' catalogues and local restaurant menus, we confirmed that the freshwater fish species of commercial interest, and therefore intended for human consumption, were sampled in this study. However, a high variability in the number of specimens collected per species in the different lakes is evident, and a scarce number was collected for some species (Table 1). In fact, only in Bracciano Lake, where the fish were purchased by a local fishmonger, we were able to collect ~30 specimens per each of the collected species. On the contrary, for all the other lakes where the sampling was commissioned to the local fishermen, the number of sampled specimens was highly variable.

3.2. Parasitological and molecular analysis

A total of 163 visible parasite nematodes were found. The nematodes were isolated only from two species – European perch (*P. fluviatilis*) and Largemouth black bass (*M. salmoides*) – out of the 17 species examined, caught both in Bolsena and Bracciano lakes (Latium). However, only a single larva isolated from the belly flaps of a European perch specimen caught in Bracciano Lake showed morphological characteristics referable to the genus *Eustrongylides* spp. The identification was confirmed by molecular analysis. Indeed, the BLAST analysis conducted using the sequence obtained from that larva allowed to attribute to the species *E. excisus*, the

only one so far reported in Italy [8,9,18,22,26].

All the other 162 nematodes found at the visceral level in European perch and Largemouth black bass were not morphologically referrable to *Eustrongylides* spp. larvae and displayed the following macroscopic morphological features: length of around 5 mm and pinkish color, with an extremity of more intense coloration, tending toward orange/brown. Subsequent observation by stereomicroscope and optical microscope revealed a significant state of degradation of the isolates, making morphological identification at the genus level unviable. A subsample of 16 nematodes (~10% of those found at visceral level) were submitted to molecular analysis. The BLAST analysis retrieved identity values ranging from 100 to 97.80% with sequences deposited for the species *Camallanus hypophthalmichthys*. This species was isolated and described since the 1960s from numerous freshwater species [37,38]. The genus *Camallanus* includes several non-zoonotic species inhabiting the digestive tract of fish species mostly belonging to Cypriniformes and Perciformes orders [5]. Therefore, the presence of this species is only reported for completeness, but the rest of the manuscript is only focused on *Eustrongylides excisus*.

3.3. Occurrence of *Eustrongylides excisus* in freshwater fish species caught in six lakes from central Italy (tuscany and latium regions)

To provide a comprehensive picture of the epidemiology of *Eustrongylides* spp. in the freshwater fish species collected in the present study, our results were analyzed considering any report of infection available in Italy or other countries (see Table 2). Moreover, the species feeding habits were also discussed, as they play a crucial role in determining the possibility of infection with the nematode larvae [8,26].

3.3.1. Positive species: European perch from Bracciano Lake

The European perch was the only species among those analyzed in this study in which a *E. excisus* larva was isolated. Therefore, the present study describes for the first time the presence of *E. excisus* in the Bracciano Lake (Latium) with a P = 3.3% (95% CI 0.01–0.17), a MA = 0.03 and a MI = 1. Several studies describing the infection of this species in other Italian lakes are available. Most of them involved fishes from the Trasimeno Lake (Umbria) and reported widely varying prevalence values. Specifically, Dezfuli et al. [21] reported a P = 6.1%, Branciari et al. [23] and Agnetti et al. [24] reported a P = 6.8% and = 6.9%, respectively, and lastly, Franceschini et al. [25] reported a prevalence ranging from P = 4.2% in 2016 to P = 68% in 2021 (P was calculated analyzing only the fillets of the collected specimens). Other Italian lakes in which the infection of this species with *Eustrongylides* spp. was documented are San Michele Lake in Piedmont (P = 10%) [6], Garda Lake in Veneto (P = 2%) [26] and Annone Lake (P = 9.6%) in Lombardy [22]. The current

Table 2
Sampled species divided according to their family, and related reports of infection with *Eustrongylides* spp. in Italy and worldwide.

Family	Species	Reports of *Eustrongylides* spp. infection			
		Italy	References	International	References
Percidae	European perch (*P. fluviatilis*)	Trasimeno Lake Lake San Michele Lake Garda Lake Annone Bracciano Lake[a]	[6,21–26]	Caspian Sea (Turkey) Lake Victoria (Romania)	[39,40]
	Pumpkinseed (L. *gibbosus*)	Trasimeno Lake Lake San Michele Lake Garda Massaciuccoli Lake	[6,8,25,26]	N.R.	[54,56]
	Pike-perch (*S. lucioperca*)	N.R.	–	Danube–Tisa–Danube Canal (DTD) (Serbia) Derbent Dam Lake (Turkey) Lake Egirdir (Turkey) Dnepr River (Ukraine) Lake Marmara (Turkey) Razim Lake (Romania)	[19,54,56,63–65]
Centrarchidae	Largemouth black bass (*M. salmoides*)	Trasimeno Lake Lake San Michele Lake Garda Lake Massaciuccoli	[6,8,23,25,26]	N.R.	–
Ictaluridae	Channel catfish (*Ictalurus punctatus*)	N.R.	–	Erie Lake (Ohio)	[47]
Cyprinidae	Tench (*Tinca*)	Trasimeno Lake	[25]	N.R.	–
	Chub (*S. cephalus*)	N.R.	–	Susurluk (Turkey)	[46]
Esocidae	Pike (*E. lucius*)	N.R.	–	Dnepr River (Ukraine) Caspian Sea (Iran) Caspian Sea (Turkey)	[39,55,63]
Atherinidae	Big-scale sand smelt (*A. boyeri*)	Trasimeno Lake Massaciuccoli lake	[8,9,14,25]	Lake Iznik (Turkey) Lake Oneida (New York) Egirdir Lake (Turkey)	[49,50,66]

[a] European perch infection with *Eustrongylides* spp. is reported in the present study. N.R.: Never Reported.

known distribution of this parasite in Italy is depicted in Fig. 2. As regards international studies, this species was found as host of *Eustrongylides* spp. both in Turkey with a P = 33.3% [39] and Romania [40]. The dietary habits of the European perch (*Perciformes* order, Percidae family) were recently described: during its development this species switches from a predominantly benthivorous diet (based on annelids, including oligochaetes) to a carnivorous diet, which mainly consists of other fish. This dietary change has been related to the inverse correlation observed between the fish size and the MI of infection, plausibly related to the change of the source of *Eustrongylides* spp. infection, oligochaetes in the case juvenile fish, and other infected fish for adult ones [26]. Accordingly, the low MI found in this study (MI = 1) could be related to the large size of the samples collected in Bracciano Lake, that were purchased from a local fishmonger. In fact, only samples of commercial size and intended for consumption were available. However, according to Menconi et al. [26], to obtain more accurate quantitative descriptors of the parasite population for this species, the sampling should also include smaller individuals. Fig. 3 depicts the most common freshwater fish species sold around Bracciano Lake (author's note), which were also the ones sampled in this study, sorted according to their trophic level. It is possible to notice that species for which reports of infection with *Eustrongylides* spp. are available in the literature, are located higher up in the chart, while negative species fall further down. Big-scale sand smelt is located around the middle of the chart, and for the species whose trophic level is lower than Big-scale sand smelt's (=3.2 ± 0.36 according to *fishbase. se*), the occurrence of *Eustrongylides* spp. has been ever documented. Thus, it is possible to hypothesize that species above this trophic level may serve as indicators of the nematode's presence in lacustrine ecosystems, as they are more susceptible to become infected with *Eustrongylides* spp. This positive correlation between the trophic level and the presence of *Eustrongylides* spp. larvae in a certain fish species is further confirmed by the fact that the only nematode detected in the samples collected from Bracciano Lake was isolated from the species with the highest trophic level (=4.4 ± 0.0) among the six collected, the European perch. The nematode presence could be related to the fact that the main definitive host of this nematode, the Great Cormorant (*P. carbo*) [22], is often observed wintering around Bracciano Lake (https://www.parcobracciano.it/area-protetta/fauna/). In order to provide a more accurate epidemiological picture of the infection with *E. excisus* of the fish species inhabiting Bracciano Lake, it would be desirable to extend the sampling activity to non-commercial species, giving priority to "indicator species", as those with a higher trophic level.

Fig. 2. Current known distribution of *Eustrongylides* spp. in Italy. Place markers indicate lakes where fish species were analyzed for the detection of *Eustrongylides* spp. larvae: red markers are placed on lakes where positive fish species were found, while green markers are placed on lakes where all examined fish were negative.

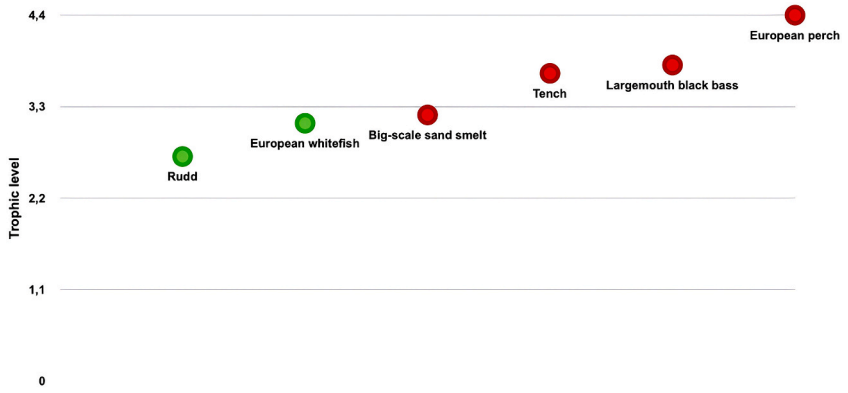

Fig. 3. Fish species locally marketed in Bracciano Lake, sorted according to their trophic level. The red color indicates species for which reports of infection with *Eustrongylides* spp. are available in the literature, while the green color indicates species for which no report is available.

3.3.2. Negative species

All the other 16 species analyzed in this study (see section 3.4.1) were found negative for the presence of *Eustrongylides* spp. larvae. However, the number of caught specimens was <15 for Pike-perch, Largemouth black bass, Rudd and Italian riffle dace caught in Bilancino Lake, Goldfish caught in Chiusi Lake, Chub, Goldfish and Freshwater bream caught in San Cipriano Lake and Pike, Tench and European whitefish caught in Bolsena Lake. Clearly, the sampling size influences the statistical reliability of the obtained data. As for the following 9 species, European whitefish, Rudd, Common bleak, South European roach, South European nase, Italian riffle dace, Goldfish and Freshwater bream, belonging to the *Salmonidae*, *Leuciscidae* and *Cyprinidae* families, the results obtained in this study are in line with the ones available in the literature. In fact, no report of infection in these species is available nor in Italy neither in other countries. The lack of infection is probably related to their algivorous, herbivorous, or insectivorous feeding habits [41–43]. Also, Tench (*Tincidae* family) and Chub (*Leuciscidae* family) could be included among the non-susceptible species, as they feed mainly on plant material and zooplankton [44,45]. These species can eventually be considered as accidental hosts, acquiring infection in heavily contaminated environments [25,46].

With regard to Channel catfish (*Ictaluridae* family), Menconi et al. [6] analyzed specimens caught in Lake San Michele (Piedmont, Italy) and did not detect any *Eustrongylides* spp. larva. The same species was investigated in Lake Erie (Ohio, USA) by Cooper et al. [47], who reported a P = 0.02%. This species has a predominantly algivorous diet, but occasionally feeds on benthic insects and small fish [48], and this would be consistent with the rare occurrence of larvae of the genus *Eustrongylides* in this species.

As regards the Big-scale sand smelt (*Atherinidae* family), no *Eustrongylides* spp. larvae were detected in this study. This finding contrasts with other studies reporting the occurrence of *Eustrongylides* spp. in this species in Italian lakes. Prevalences ranging from 0.06% to 40% in the Trasimeno Lake [14,25], and 2.3% and 5.1% in Massaciuccoli Lake [8,9] were described. The presence of *Eustrongylides* spp. in this species is also reported in other countries, in particular in the USA (P = 5.1%) and in Turkey (P = 6.6% in Lake Iznik and P = 5.1% in Lake Egirdir) [49,50]. The Big-scale sand smelt is an opportunistic carnivore, capable of exploiting the available resources and feeding in cold months on benthic animals (such as infected oligochaetes) while in warm months on plankton [49]. However, this seasonality was not observed in Italy so far [8,25]. The negativity of Big-scale sand smelts sampled in Bolsena and Bracciano lakes is probably related to the fact that, according to our results, *Eustrongylides* spp. is not present in Bolsena Lake, while in Bracciano Lake its presence was found to be still limited to strictly carnivorous species, such as the European perch. Therefore, it is possible that the Big-scale sand smelt, which feeds mainly on detritus and zooplankton [51], does not acquire the infection in these conditions, or that the prevalence value is very low.

Reports describing *Eustrongylides* spp. infection in Pumpkinseed (*Percidae* family) and Largemouth black bass (*Centrarchidae* family) are only available for Italy. Pumpkinseed was in fact investigated in Lake Trasimeno by Franceschini et al. [25], who analyzed only fillets and reported a P varying from 47% in 2016 to 99% in 2021. The same species was also investigated in Lake San Michele and Lake Garda, reporting a prevalence of 18.3% and 3.7, respectively [6,26]. Ultimately, a P of 4.6% for this species was also reported in Lake Massaciuccoli [8]. Pumpkinseed specimens analyzed in this study were all collected in Bilancino Lake, in which *Eustrongylides* spp. was not found. Largemouth black bass was initially investigated in Trasimeno Lake, San Michele Lake, Garda Lake, and the reported prevalences were 1.89 %, 16.7%, and 25%, respectively [6,23,26]. More recent studies conducted on fillets of Largemouth black bass specimens from Trasimeno Lake and on whole specimens from Massaciuccoli Lake reported a prevalence ranging from 0.18 to 0.47 for the first one, and of 25% for the second [8,25]. Differences in the prevalence values reported in the literature may be related to the portion of the fish (fillet or whole fish) and to the number of specimens analyzed. In this study, Largemouth black bass specimens were collected from Bilancino, Bolsena and Bracciano lakes, and no *Eustrongylides* spp. larvae were detected after the parasitological analysis. As no positive fish species were observed in the first two lakes (Bilancino, Bolsena) and nor any reports attesting *Eustrongylides* spp. presence is available, the negativity of Largemouth black bass specimens can be ascribed to the absence of the parasite in these basins. As for Bracciano Lake, the results of this study suggest that the infection of resident fish species with *Eustrongylides* spp. larvae is currently very low. Therefore, the lack of infection in this species can be ascribed to its low trophic level and to the fact that only the top predator species seem to acquire the infection in environments with reduced *Eustrongylides* spp. presence. Pumpkinseed and

Largemouth black bass share common feeding habits, especially at juvenile age, as both feed on benthic invertebrates (such as infecting oligochaetes). In adulthood the diet of the Pumpkinseed does not undergo any remarkable changes, while Largemouth black bass begins to feed on a wide variety of other fish, potentially becoming a paratenic host for *Eustrongylides* spp. larvae [52,53].

Eustrongylides pp. infection of Pikeperch (*Percidae* family) and Pike (*Esocidae* family) species has not been so far investigated in Italian lakes. This is, in fact, the first study conducted in Italy in which these two species were analyzed. The lack of detection of *Eustrongylides* spp. larvae can be probably related, other than to the absence of *Eustrongylides* spp. in Bilancino and Bolsena lakes, where both species were solely collected, to the limited number of the specimens sampled: 11 for Pikeperch and only one for Pike, respectively. Instead, records of *Eustrongylides* spp. infection are available in Eastern Europe and Turkey, with prevalences ranging from 14.3% to 100% for Pikeperch and 5%–90% for Pike [39,54–56]. The positivity in these species may be motivated by their strictly carnivorous feeding habits [57,58], as they feed mostly on other fish and may act as paratenic hosts.

3.4. Implications for local fishery supply chains related to the presence of Eustrongylides spp.

To fully understand the implications for public health and fishery products quality associated with the presence of *Eustrongylides* spp. in freshwater ecosystems, and by consequence, in the inhabiting fish species, a combination of factors must be considered. The first consideration is whether the fishing activity is recreational ("*no-kill*") or intended for consumption. If fishery products are intended for consumption, it is necessary to know if they are of commercial interest, and to characterize the typical patterns of distribution, preparation, and consumption. Finally, for each species of interest, it is necessary to assess whether reports of infection with *Eustrongylides* spp. are available in the literature. In this way we can sort species which present a higher risk to host nematode larvae, from species whose feeding habits, together with the absence of infection reports, make the risk negligible.

3.4.1. Risk associated to fish species caught in lakes in Tuscany

Lacustrine areas in Tuscany are not characterized by the presence of a freshwater fishery supply chain, while sport and recreational fishing is mainly practiced. This is the case of Bilancino, Chiusi, San Cipriano and Montedoglio lakes, in which fishermen that practice recreational fishing are required by law to release their catches [59]. Despite this, it cannot be ruled out that unauthorized fishing, and subsequent consumption of potentially contaminated fishery products, could happen. All the fish species collected in the aforesaid Tuscan lakes in the present study were not infected by *Eustrongylides* spp. larvae, leading to the conclusion that the nematode is currently not present in these areas. However, the reliability of the data regarding the absence of *Eustrongylides* spp. larvae in the fish species sampled in the Tuscan lakes must be put in relation to the number of individuals sampled for each species considered. In fact, the number of individuals of each species caught in the Tuscan lakes was variable, because the catches made by the fishermen were not aimed at sampling a predetermined number of individuals belonging to target species, but at sampling as wide a variety of fish species as possible from each lake. Consequently, for some species, the sampling size was very limited, reducing the statistical consistency of the results obtained from the analysis of these species. The 13 species analyzed (*P. fluviatilis, L. gibbosus, S. lucioperca, M. salmoides, I. punctatus, S. cephalus, S. erythrophthalmus, A. alborella, S. rubilio, P. genei, T. muticellus, C. auratus, A. brama*) (Table 1) show differences in their feeding habits, which contribute to the degree of susceptibility to the infection with *Eustrongylides* spp. In fact, for detritivorous, herbivorous, and insectivorous species (*S. erythrophthalmus, A. alborella, S. rubilio, P. genei, T. muticellus, C. auratus, A. brama*) no positivity to the infection is reported in the literature. Moreover, for species whose diet only sporadically includes zooplankton (Chub, Tench) or other fish (Channel catfish) the infection reports documented in the literature are very sporadic and the authors report low, almost negligible prevalences (Table 2). On the contrary, a higher susceptibility can be hypothesized for the carnivorous species collected in the Tuscan lakes (*P. fluviatilis, L. gibbosus, S. lucioperca, M. salmoides*) (see section 3.4.2). In fact, even if the parasite appears not to be present, a future spread of *Eustrongylides* spp. towards these areas cannot be excluded. The geographical proximity to the Massaciuccoli Lake area (Province of Lucca), where the nematode has already been detected in some fish species that were also sampled from other lakes investigated during this study (*L. gibbosus* and *M. salmoides*), should be considered. Indeed, populations of Great cormorants may settle around these lakes moving from nearby areas. Therefore, the presence of fish species whose susceptibility to the infection has been widely documented would allow *Eustrongylides* pp. to complete its biological cycle and establish itself in the lake.

Considering all the currently available data in the framework of a qualitative risk assessment, as proposed by Crotta et al. [60], for the Tuscany lakes investigated in this study it could be stated that the likelihood that an infected fish reaches a consumer is negligible, with a high level of uncertainty due to the scarcity of available data.

However, recreational fishing associations and their members should be provided with information about the biology and risks associated with *Eustrongylides* spp. This should be done both to gather useful reports and to inform on the health issues that the consumption of contaminated products would pose.

3.4.2. Risk associated to fish species caught in Latium Lakes

The situation pertaining to the Latium lakes investigated in this study (Bolsena Lake, Bracciano Lake) is different from the previous one both in terms of the type of fishing and destination of fishery products. In fact, around both lakes, a strong tradition related to fishing and marketing of local fishery products exists. In these areas fisherman cooperatives and associations, numerous fishmongers selling locally sourced fish and small restaurants that prepare and serve these products exist (author's note). Among the most consumed freshwater fishery products, the European perch is highly appreciated, holds a certain economic importance [25] and it's common to be found at fishmongers and restaurants (author's note). Thus, the presence of *E. excisus* in the fillet of a European perch specimen purchased by a fishmonger near Bracciano Lake suggests the need to establish risk management measures for fish caught in

this lake. Also fish from Bolsena Lake should be considered at risk for the presence of *Eustrongylides* spp., as its close geographical proximity to areas where it has already been found, particularly the Trasimeno Lake [18,25], could favor the migration and subsequent settling of infected Great cormorants from these areas. In addition, the three most sampled fish species in Lake Bolsena (European perch, largemouth bass and largemouth smelt, with 30, 24 and 270 specimens respectively) are all frequently reported in Italy and abroad for the presence of the nematode [25,26,39] (See Table 2). They can play both the role of secondary host, in the case of juvenile European perch and largemouth bass, and paratenic host, in the case of adult European perch and Pike [8,26,56]. Thus, framing such results in the context of a qualitative risk assessment, in this case the risk that an infected fish may reach a consumer cannot be considered negligible, with a low level of uncertainty.

Therefore, appropriate risk management measures should be implemented at each step of the food chain in Latium region, and FBOs which work in fishing, marketing, and catering, should receive adequate training as regards the presence of *Eustrongylides* spp. larvae. The following measures should be systematically applied to the species characterized by a high trophic level, or otherwise omnivorous or carnivorous feeding habits that would justify the increased likelihood of infection with *Eustrongylides* spp. larvae. Those of major commercial interest should be particularly considered, and stronger efforts should preferentially be directed toward those species for which infection with *Eustrongylides* spp. has already been documented in Italy or in other countries. The first measure to be applied by fishermen with the aim of reducing/nullifying the parasite load, is gutting. As prescribed by Regulation (EC) No 853/2004 [30], "*where gutting is possible from a technical and commercial viewpoint, it must be carried out as quickly as possible after the products have been caught or landed*". This measure can be systematically applied only on large specimens and enables the FBO to physically remove the larvae located at visceral level, and also prevents their migration, which can occur both *intra-vitam* and *post-mortem* [21,24]. Regulation (EC) No 853/2004 [30] prescribes to submit fishery products to visual Inspection of both the product's surface and coelomic cavity "*for the purpose of detecting visible parasites before being placed on the market*". When visual inspection cannot be applied to all individuals, such as when dealing with batches of small species, it can be applied to a "*representative number of samples*", as prescribed by Regulation (EC) No 2074/2005 [33]. Moreover, visual inspection can be repeatedly applied along all steps of the supply chain, increasing the chance of detecting visible parasites in fishery products. In fact, FBOs operating in fishmongers can perform the search for larvae by visual inspection, both on the whole fish and during the following processing steps such as filleting. In addition, for species in which high prevalences are documented in the literature, such as the European perch, *belly flap* trimming may also be effective in reducing the parasite load [25]. Also catering services that handle local fishery products should perform visual inspection of the fillets to detect any encysted larvae at the muscular level, and eventually to manually remove them. Ultimately, before being served, fishery products should always undergo appropriate thermic treatment, as prescribed by Regulation (EC) No 853/2004 (cooking at 60 °C at core for 10 min) [30]. Recent studies on the resistance of *Anisakis* spp. to heat treatments highlighted how the sensitivity of this nematode can be strongly influenced by its thermal history and by the stress induced by previous to treatments, (e.g salting). Based on these studies, it was hypothesized that anisakids larvae have variable resistance to thermal treatments, but that in any case cooking at 60 °C for 10 min is sufficient to inactivate the nematode [32]. It should be noted that freshwater fishery products are often consumed in the form of carpaccio, tartare or other preparations that do not involve heat treatments and high temperatures, but only slight marinades [14]. For products that are intended to undergo such mild treatments, as well as for products intended to be consumed raw or almost raw, Regulation (EC) 853/2004 [30] requires these products to be subjected to freezing at −20 °C at core for not less than 24 h. The application of such a freezing treatment would therefore be essential to inactivate any parasites missed at the visual inspection. Currently, no specific trials have been performed to understand the susceptibility of the *Eustrongylides* spp. larvae to thermal treatments [8]. Moreover, the FDA's Fish and Fishery Products Hazards and Controls guidance suggests that treatments such as pickling and brining can reduce, but not eliminate, the parasitic risk in seafood products, including the one associated with *Eustrongylides* spp [61]. Finally, since consumers may purchase local fishery products directly from fishmongers, it is necessary to provide them with an appropriate training about the risks related to the nematod''s presence in freshwater fishery products and on how to decontaminate these products, with the aim of giving them the necessary knowledge to manage the risk associated with *Eustrongylides* spp. larvae at the household level. In order to protect consumers' health, the Ministerial Decree of the Italian Ministry of Health issued on July 17, 2013 [62], prescribes that fishery products "*to be consumed raw, marinated or not fully cooked […] must first be frozen for at least 96 h at -18°C in a domestic freezer*". This measure, which is historically linked to the management of the risk associated with anisakid nematodes, could be applied with the aim of preventing the infections caused by the presence of *Eustrongylides* spp. larvae in freshwater fishery products for domestic consumption as well.

4. Conclusions

This study contributes to broadening the knowledge on *Eustrongylides* spp. distribution in six lakes from central Italy that had not been investigated before. No *Eustrongylides* spp. larvae were found in fish samples caught in the four lakes of Bilancino, Chiusi, Montedoglio, and San Cipriano (Tuscany) and in Lake Bolsena (Latium), suggesting that the parasite is currently not present in these areas. To strengthen this assumption, it would be desirable to collect a higher number of specimens for each fish species. This aspect appears to be more relevant for those species for which a lower number of specimens was collected. On the contrary, the finding of only one larva of *E. excisus* in one specimen of European perch collected in Bracciano Lake adds another Italian basin to the list of the ones in which genus *Eustrongylides* spp. has been reported. The presence of the nematode in this area is likely a consequence of the increase in wintering and nesting sites of its definitive host, the Great cormorant (*P. carbo*), often seen in that area. Moreover, factors related to dynamics in the first intermediate hosts population, including alterations of water characteristics, could also play a role. Since a structured supply chain of freshwater fishery products exists around Bracciano Lake, FBOs must apply adequate measures to manage the risk associated with the presence of *Eustrongylides* spp. in these products. The risk management measures suggested in this paper are

intended to protect both consumers' health, as well as local economies, which could be harmed if dangerous and unfit for consumption products are placed on the market. The monitoring activity should preferably be directed toward the most susceptible fish species that, due to their feeding habits, tend to accumulate *Eustrongylides* spp. larvae in their viscera and muscles. Nowadays, these processes are still in their early stages, since the occurrence of the nematode is still poorly documented or not documented at all in the investigated geographical areas.

CRediT authorship contribution statement

Marta Di Maggio: Writing – original draft, Investigation, Data curation. **Miriana Coltraro:** Investigation, Data curation. **Lara Tinacci:** Writing – review & editing, Investigation, Data curation. **Lisa Guardone:** Writing – review & editing, Data curation. **Enrica Ricci:** Methodology, Investigation. **Carlo Corradini:** Investigation. **Francesca Susini:** Funding acquisition, Conceptualization. **Andrea Armani:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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