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Eustrongylides excisus in fish species caught in the Massaciuccoli Lake (Northwest Tuscany, Italy): Implications for freshwater fish quality and public health

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ABSTRACT

In recent years, nematodes belonging to the genus *Eustrongylides* spp. have been increasingly reported in Italian lakes and in several freshwater fish species. This work aimed to investigate the occurrence of *Eustrongylides* spp. in 11 freshwater fish species collected from Massaciuccoli lake (Northwest Tuscany, Central Italy): Black bullhead, *Ameiurus melas* (Rafinesque, 1820), Goldfish, *Carassius auratus* (Linnaeus, 1758), Wels catfish, *Silurus glanis* (Linnaeus, 1758), Thinlip grey mullet *Chelon ramada* (Risso, 1827), Pumpkinseed, *Lepomis gibbosus* (Linnaeus, 1758), Common carp, *Cyprinus carpio* (Linnaeus, 1758), Tench (*Tinca tinca*) (Linnaeus, 1758), European eel, *Anguilla anguilla* (Linnaeus, 1758), Largemouth black bass, *Micropterus salmoides* (Lacepède, 1802), Big-scale sand smelt, *Atherina boyeri* (Risso, 1810), and Stone moroko, *Pseudorasbora parva* (Temminck & Schlegel, 1846). Overall, 4053 fish specimens (327 large and 3726 small specimens), collected in eight different samplings (July 2020–April 2021), were visually examined, and subjected to artificial digestion. A total of 476 nematode larvae were collected and microscopically identified as *Eustrongylides* spp. A subsample (10%) of the collected larvae was subjected to molecular analysis through the analysis of the ITS gene region and identified as *E. excisus*. Quantitative descriptors of the parasite population were calculated using the data collected from the six species that tested positive (*A. melas*, *S. glanis*, *L. gibbosus*, *M. salmoides*, *A. boyeri*, *C. ramada*) and discussed in the light of their dietary habits. This study reports for the first time the presence of *E. excisus* in *S. glanis* and juvenile of *C. ramada* in Italy. The apparent expansion of this zoonotic parasitic nematode in freshwater fish species, possibly related to changes in the lakes' ecosystems, could represent an issue for local economies. Therefore, Control Authorities and Food Business Operators must respond to this emerging hazard with appropriate control measures, to prevent potentially unfit or dangerous products from reaching the consumer.

1. Introduction

The increased consumption of raw or undercooked fish products in Western countries in recent years may expose consumers to the risk of parasite infection. Parasites affecting freshwater fish species are potentially responsible for serious diseases in humans (Chai et al., 2005 Scaramozzino et al., 2018; Pozio et al., 2013; Sholz et al., 2009).

The genus *Eustrongylides* (Jägerskiöld, 1909) (Dioctophymatidae family) has a cosmopolitan distribution (Northern and Southern America, Europe, Asia) (Guagliardo et al., 2019; Honcharov et al., 2022; Mazzone et al., 2019; Metin et al., 2014; Xiong et al., 2013) and a complex and indirect life cycle, involving a wide range of fish species and fish-eating birds inhabiting freshwater ecosystems. Ichthyophagous birds act as definitive hosts, oligochaetes (*Annelida* phylum) as first intermediate hosts and bentivorous and planktivorous fish as second intermediate hosts. In the second intermediate host the third-stage larva

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moult into the fourth stage (L4) and remain as L4 until ingestion by the definitive host (Measures, 1988a; Moravec, 2013; Spalding & Forrester, 1993, 2008). L4 larvae can also accumulate in predatory fish species, serving as paratenic hosts (Honcharov et al., 2022; Measures, 1988a). The biological cycle of *Eustrongylides* spp. is summarized in Fig. 1.

Humans represent accidental hosts, following the consumption of infected raw or undercooked freshwater fish, which may act as second intermediate or paratenic hosts within the parasite cycle (Food and Drug Administration, 2012, 2022). Infections by larvae of the genus *Eustrongylides* are sporadic and, overall, only seven human cases have been described worldwide so far (five in the USA and two in South Sudan). In the USA, four cases were due to the ingestion of live minnows used as bait for fishing, while the remaining one was attributed to the consumption of domestically prepared sushi and sashimi (Centers for Disease Control, 1982; Eberhard et al., 1989; Wittner et al., 1989). The other two documented cases, from South Sudan, appear to be a cutaneous form of parasitosis, but it was not possible to identify the source of infection (Eberhard & Ruiz-Tiben, 2014). In all ascertained Eustrongylidosis human cases the parasites were identified as L4 (Guardone et al., 2021).

No human cases have been reported so far in Europe, and the presence of these parasites in definitive and intermediate hosts has so far been little investigated, although it has recently attracted increasing attention (see Table 1 in Honcharov et al., 2022). In Italy, the first record of the genus *Eustrongylides* occurred in 2015, from the Trasimeno Lake (Umbria region, Central Italy) (Dezfuli et al., 2015). Thereafter, it has been reported in the same lake (Agnetti et al., 2016, 2019; Branciari et al., 2016; Franceschini et al., 2022; Mazzone et al., 2019) and in different lakes located in Central and Northern Italy: Lake San Michele in Piedmont (2020), Lake Garda (2021) and Lake Annone (2022) in Lombardy, Lake Massaciuccoli (2021) in Tuscany and informally in some Alpine lakes (Ceresio and Montorfano) (Menconi et al., 2020, 2021; Guardone et al., 2021; Rusconi et al., 2022).

The different species of *Eustrongylides* described so far *(E. excisus, E. ignotus, E. mergorum, E tubifex)* display distinctive morphological features as adults, while larval stages are morphologically indistinguishable and molecular identification is required (Mazzone et al., 2019; Measures, 1988a; Xiong et al. 2009). Mazzone et al. (2019) was the first to describe adult *E. excisus* from the great cormorant (*Phalacrocorax carbo*), Lake Trasimeno (Italy) using morphological and molecular approach. All subsequent studies conducted at the national level have identified *Eustrongylides* spp. larvae (Franceschini et al., 2022; Guardone et al., 2021; Menconi et al., 2021) based on the comparison of molecular sequences generated in Mazzone et al. (2019). The larvae implicated in human cases worldwide were not molecularly identified (Guardone et al., 2021) and, therefore, the zoonotic potential and the geographical distribution of the different species belonging to the genus *Eustrongylides* still needs to be fully described (Guardone et al., 2021).

Fig. 1. *Eustrongylides* spp. biological cycle.

Table 1
Fish species included in the study, with number of fish specimens and detail of the number (No) of parasites detected in positive species by visual inspection, by enzymatic digestion (of viscera and belly flaps) and by axial muscle incision. NE: not examined. Species shaded in grey were found to be negative.

	Fish species	Number of fish specimens	No of parasites detected by visual inspection	No of parasites detected by enzymatic digestion		No of parasites detected by axial muscle incision	Total No of parasites
				Viscera	Belly flaps		
Large specimens	Black bullhead (*Ameiurus melas*)	77	15	7	16	0	38
	Goldfish (*Carassius auratus*)	76	–	–	–	–	0
	Wels catfish (*Silurus glanis*)	56	118	95	28	–	241
	Thinlip grey mullet (*Chelon ramada*)	48	–	–	–	–	0
	Pumpkinseed (*Lepomis gibbosus*)	23	–	1	–	–	1
	Common carp (*Cyprinus carpio*)	21	–	–	–	–	0
	European eel (*Anguilla anguilla*)	17	–	–	–	–	0
	Tench (*Tinca tinca*)	5	–	–	–	–	0
	Largemouth black bass (*Micropterus salmoides*)	4	–	–	1	–	1
Total large specimens		**327**					**281**
Small specimens	Big-scale Sand smelt (*Atherina boyeri*)	3500	112	68		NE	180
	Small Thinlip grey mullet (*Chelon ramada*)	210	11	4		NE	15
	Stone moroko (*Pseudorasbora parva*)	16	–	–		NE	0
Total small specimens	**3726**						**195**
Overall total	**4053**						**476**

Food Business Operators (FBOs) carrying out any stage of production, processing and distribution of food shall comply with general and specific hygiene requirements (Regulation (EC) No 852/2004 and Regulation (EC) No 853/2004). Regarding fishery products, the Regulation (EC) No 853/2004 provides the requirements for parasite checks, stating that it is up to FBOs to carry out their own checking during all production stages, in accordance with the Commission Regulation (EC) No 2074/2005. A visual inspection shall be performed to detect visible parasites and to avoid contaminated fish products being sold for human consumption. The larval stage of *Eustrongylides* spp. has an unmistakable morphological appearance. It is in fact characterized by a bright pink-red color and considerable size (3–5,5 cm in length and 0,5-1 mm in diameter) (Measures, 1988a). Therefore, *Eustrongylides* spp. larvae perfectly fit with the definition of "*visible parasites*" defined by the Codex Alimentarius Commission "*nematodes longer than 1 cm or parasites with a capsular diameter of at least 3mm*" (Codex Alimentarius Commission, 1971), as well as with the definition given by Commission Regulation (EC) No 2074/2005: "a *parasite or a group of parasites which has a dimension, colour or texture which is clearly distinguishable from fish tissues*". Independently from the relative parasite zoonotic potential, fish products obviously contaminated with visible parasites are not fit for human consumption (Regulation (EC) No 178/2002). Indeed, the repulsive appearance of fish contaminated by large nematodes such as *Eustrongylides* spp. can provoke rejection in consumers and damage the seller's reputation even if the parasite is dead (D'Amico et al., 2014; Branciari et al., 2016; Mazzone et al., 2019; Franceschini et al., 2022). Therefore, *Eustrongylides* spp. must be considered among the hazards/defects potentially affecting freshwater fish species intended for consumption and, preventive measures specifically developed according to the fish species, larvae localization and type of products, have been proposed to avoid the commercialization of products at risk by FBOs (Franceschini et al., 2022).

The aim of this study, as a follow-up of the research conducted by Guardone et al., in 2021, was first to identify *Eustrongylides* spp larvae found in fish of Lake Massaciuccoli using molecular methodology and then to discuss the possible consumer health risks through a critical evaluation of current food safety regulations and fish culinary uses. Overall, epidemiological data produced in this study, discussed together with those of published literature, will be useful for assessing the potential expansion of *E. excisus* in the investigated region and establishing proper risk management procedures for FBOs.

2. Materials and methods

2.1. Sampling, parasitological examination and identification

2.1.1. Sampling

The sampling was carried out monthly from July 2021 to April 2022 (except for October and January), for a total of eight samplings. Each sampling was carried out in co-operation with the Local Health Authority (LHA), and with fishermen. Fishermen were asked to provide the widest possible variety of fish species. Once caught, the specimens were transferred within a few hours to the laboratory where they were immediately processed or stored in a freezer at −20 °C until analysis. The FAO FishBase database (https://www.fishbase.se/home.htm#) was used as official source for specimens morphologically identification (Table SM1). Only in the case of *Chelon* spp. specimens, for which the morphological features were not diagnostic, a molecular identification was performed according to the protocol described by Tinacci et al. (2018). A unique alphanumerical code was attributed to the specimens, which were photographed, measured, and weighed (as single specimens or as pools of ten specimens, see section 2.1.2) before undergoing parasitological examination. All data was recorded on an Excel file.

2.1.2. Parasitological analysis

The sampled fish species were analyzed as a single specimen (large

specimens) or as a pool of specimens (small specimens). The following steps were performed for each individual large specimen: weighing and length measurement of the entire specimen, evisceration, evaluation by visual inspection of the abdominal cavity and viscera (in particular, of the liver, gonads, and serosae) (Fig. 2A), weighing of viscera, removing and weighing of abdominal muscles (belly flaps) and incision of axial muscles (at least three cuts per specimen) (Fig. 2B). All these steps were conducted in lighting conditions recommended by Commission Regulation (EC) No 2074/2005 for the detection of visible parasites. Then, a separate enzymatic digestion of viscera and belly flaps was performed, using a magnetic stirrer (digestion temperature 37–45 °C) with hydrochloric acid (HCL) and pepsin; the digestion fluid was filtered, and the sieved material analyzed to detect parasites. The digestion solution was obtained adding 50 mL of 10% HCl and 10 g of pepsin from PLYtricons® to 1 L of water (CTSV srl, Brescia). Both methods (visual and enzymatic digestion) were already used in previous studies (Guardone et al., 2019, 2020).

The small specimens were analyzed by visual inspection, followed by artificial digestion of pools of ~100 g, following the same protocol described above. All parasites found after visual inspection or digestion were collected and counted. Then, they were macroscopically identified to genus level by their characteristic bright pink-red color and considerable size (3–5,5 cm in length and 0,5-1 mm in diameter), and then observed microscopically (Nikon Eclipse E 200), considering the descriptions in Mazzone et al. (2019), Measures (1988a) and Panesar and Beaver (1979). Parasites were then stored in 70% ethanol for subsequent molecular identification.

2.1.3. Molecular identification

Molecular analysis was performed on a subsample (10%) of the parasites found in each infected species. DNA extraction, amplification of the 5.8S gene and the ITS-two region plus approximately 70 nucleotides of the 28S gene (ITS), sequencing and sequence editing were performed following the protocol described in Guardone et al. (2021). In details, a fragment of about 900-bp was amplified using the primers NC2 and NC5 (Zhu et al., 1998). PCR amplifications were set up in a 20 μl reaction volume containing 4 μl of a 5 × buffer (biotechrabbit GmbH, Berlin, Germany), 200 μM of each dNTP (dNTPmix, EurocloneS.p.A-Life Sciences Division, Pavia, Italy), 250 nM primers, 2.5 U Taq DNA Polymerase (biotechrabbit GmbH, Berlin, Germany), 1–2 μl of DNA (50–100 ng/μl) and DNase free water (Water Mol. Bio. Grade, DNase-RNase and Protease free, 5Prime GmbH, Hamburg, Germany) with the following cycling program: 95 °C for 3 min; 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 75 s; and final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 2% agarose gel. Amplicons presenting the expected length were forward and reverse Sanger sequenced. All the sequences obtained from parasites collected in this study were compared with all the ITS sequences of *Eustrongylides* spp. available in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST). Then, all the sequences obtained from the parasites isolated from each host species were aligned using BioEdit and a pairwise analysis with Kimura 2 parameter model (Kimura, 1980) was performed to verify the presence of polymorphisms. For each group of sequences (per each host) one representative sequence per specific polymorphism was deposited in GenBank. These deposited sequences, together with those used in the Maximum Likelihood (ML) analysis by Mazzone et al. (2019), Menconi et al. (2021), Guardone et al. (2021), and those produced in Franceschini et al. (2022) were used to produce a distance tree by using ML method and Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap re-samplings as described in Guardone et al. (2021). The dendrogram was obtained with MEGA-X software (Kumar et al., 2018).

2.2. Statistical analysis

The prevalence (P) (and 95% confidence intervals - CI), mean abundance (MA) and mean intensity (MI) were calculated according to Bush et al. (1997).

3. Results

3.1. Sampling and parasitological analysis

Overall, 4053 fish specimens were collected, belonging to 11 fish species. Of these, 327 were large specimens analyzed individually (see Section 3.1.1) and 3726 were small specimens, analyzed in pools (see Section 3.1.2). The number of specimens were distributed among the species as follows: 77 *A. melas* (Black bullhead), 76 *C. auratus* (Goldifish), 56, *S. glanis* (Wels catfish), 23 *L. gibbosus* (Pumpkinseed), 21 C. *carpio* (Common carp), 17 *A. anguilla* (European eel), 5 *T. tinca* (Tench), 4 *M. salmoides* (Largemouth black bass) and 3500 *A. boyeri* (Big-scale sand smelt). The specimens belonging to the genus *Chelon* were molecularly identified as *C. ramada*, and subdivided in large specimens (n = 48) and

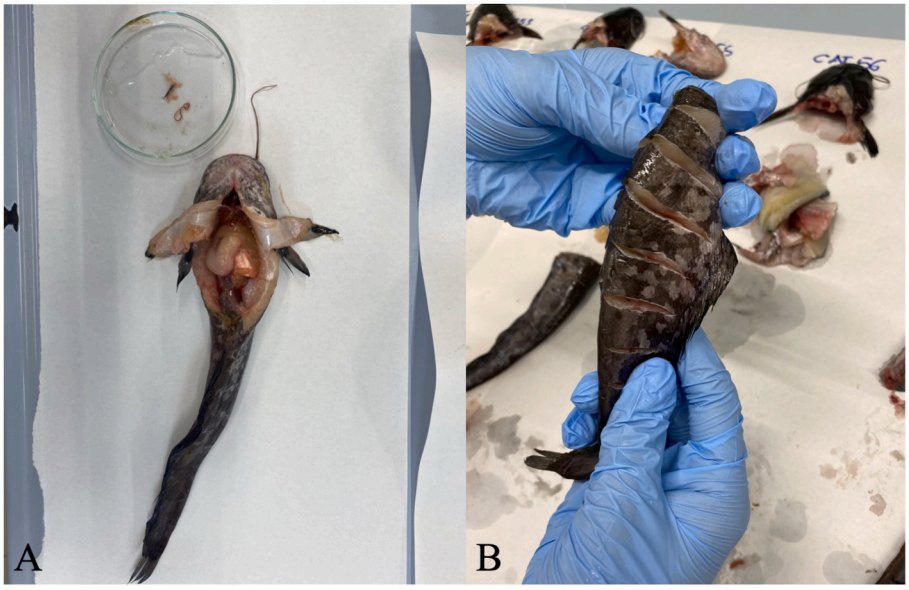

Fig. 2. Visual inspection of coelomic cavity (A) and incision of axial muscles (B) performed during the Parasitological analysis of a black bullhead (*A. melas*) specimen.

small specimens (n = 210). A total of 476 nematode larvae, all morphologically identified as belonging to the genus *Eustrongylides* spp., were found (Table 1). In particular, it was possible to differentiate larvae of two different sizes (intended as length and diameter), referable to the L3 and L4 larval forms described by Measures (1988b).

3.1.1. Large fish specimens

After the parasitological examination 281 larvae were found, distributed as follows: 241 larvae in Wels catfish specimens, 38 in Black bullhead, one in Pumpkinseed, one in Largemouth black bass (Table 1). To be noted that no larvae were found during the incision of the axial musculature, performed during the visual inspection of the large specimens. All adult Thinlip grey mullets, Goldfishes, Tenches, European eels, and Common carps tested negative.

In the Wels catfish specimens, of the 241 larvae found, 213 (88.4%) were in the viscera and coelomic cavity (118 after visual inspection and 95 after digestion), while only 28 (11.6%) were found after the digestion of the belly flaps. Of the 38 larvae found in Black bullhead specimens 15 (39.5%) were found during visual inspection of the viscera and coelomic cavity and 23 (60.5%) after the subsequent digestion of the viscera and belly flaps (seven and 16 larvae, respectively). A single larva was found in the coelomic cavity of a Largemouth black bass by visual inspection, and another one in a Pumpkinseed specimen after the belly flap digestion (Table 1).

The highest P value was found for Wels catfish (P = 75.0%, 95% CI 61.6–85.6%), followed by Black bullhead (P = 28.6% 95% CI 8.8–40.0%), Largemouth black bass (P = 25.0% 95% CI 6–80.6%) and Pumpkinseed (4.3% 95% CI 0.1–21.9). The highest MI was found in Wels catfish (5.7), followed by Black bullhead (1.7), while in both Pumpkinseed and Largemouth black bass it was 1 (Table 2).

3.1.2. Small fish specimens

The 3726 small specimens were composed of 3500 Big-scale sand smelts (belonging to six pools), 210 small Thinlip grey mullets (belonging to three pools) and 16 Stone moroko of comparable size to the Big-scale sand smelt (one pool). Each pool included all specimens of a species caught within a month. After the parasitological examination of small specimens, 195 larvae were found. Of these, 180 (92%) were found during the analysis of the six pools of Big-scale sand smelt: 112 (62%) were found after the visual inspection and 68(38%) after the enzymatic digestion. Regarding small Thinlip grey mullets, two out of the three pools tested positive leading to the collection of 15 larvae (11 after visual inspection and four after the enzymatic digestion). No larvae were found in the single pool of Stone moroko (Table 1). In the case of Big-scale sand smelt the P value (5.1%; 95% CI 4.4–5.9%) was calculated assuming a mean intensity = 1 due to the small size of this species (mean total length of about 8–10 cm) (Guardone et al., 2021; Lorenzoni et al., 2015; Çolak, 2013). The same assumption could not be postulated for the species *C. ramada*, in which more than one larva per specimen was occasionally found during visual inspection. Therefore, the only quantitative descriptor of the parasite population evaluated for this species was the mean abundance of infestation (0.07). Quantitative descriptors of the parasite population are given in Table 2.

3.2. Molecular identification

By sequencing 10% of the parasites found in each host species (Table 1) a total of 50 sequences were obtained. The BLAST analysis retrieved an identity score ranging from 100% to 97.12% with the ITS sequences available in GenBank. The lowest identity values were obtained toward reference sequences presumably not belonging to the species *E. excisus*, and not identified to species level by Xiong et al. (2013). Specifically, the BLAST analysis retrieved a 100–99.74% identity with the reference sequences produced by Mazzone et al. (2019) that currently are the only ones produced from morphologically identified adult specimens of *E. excisus* collected from the Trasimeno Lake, and a maximum identity of 100% with reference sequences deposited as *E. excisus* by Menconi et al. (2021) and Franceschini et al. (2022), as *E. aff. excisus* by Guardone et al. (2021) and as *Eustrongylides* sp. by Xiong et al. (2013) and Youssefi et al. (2020). An identity score of 100% was also found with one sequence deposited as *E. ignotus* (MK340917). Further details are shown in Table SM2. The pairwise analysis revealed the homology of the parasite sequences produced from each host except for those produced from the parasites isolated from *S. glanis*. Within this group of sequences, five different types of polymorphism were in fact identified. Therefore, a total of 10 sequences were finally deposited (GenBank accession nr. OQ359109-118) (Table 1SM). The BLAST identity scores were confirmed by the ML analysis. The ten sequences produced in this study clustered with the sequences of: 1) *E. excisus* produced by Mazzone et al. (2019); Menconi et al. (2021) and Franceschini et al. (2022); 2) *E. aff excisus* produced by Guardone et al. (2021), 3) *Eustrongylides* sp. produced by Xiong et al. (2013) (clade three); Youssefi et al. (2020), and Pekmezci and Bolukbas (2021) (Fig. 3).

4. Discussion

4.1. Molecular identification

Overall, the results of the molecular identification conducted in the present study assigned the larvae to the species *E. excisus* and confirmed the findings of Guardone et al. (2021), except for an identity score of 100% with a sequence deposited as *E. ignotus* (MK340917) produced by Jia et al. (2019). Considering that this latter represents the only ITS sequence deposited for this species, this result was further investigated to better define the origin of the parasite from which the sequence was produced. According to the authors, this sequence was produced from a L4 larva collected from a fish host (*Culter alburnus*) caught in a freshwater lake in Shanghai, and, as such, not morphologically identifiable at species level (Mazzone et al., 2019). In fact, as already mentioned, among the species currently belonging to this genus (*E. excisus, E. ignotus, E. tubifex, E. mergorum*), the latter of which (*E. mergorum*) introduced into World Register of Marine Species (WoRMS) database in 2022 (WoRMS, 2022), display distinctive morphological features as adults, while larval stages are morphologically indistinguishable and molecular identification is required (Mazzone et al., 2019; Measures, 1988a; Xiong et al. 2009). Therefore, considering that currently no sequences produced from adult specimens of *E. ignotus* are available, the sequence produced in the work of Jia et al. (2019) was considered unverified and not included in the further distance analysis, as in Shamsi

Table 2
Prevalence (P%) with 95% confidence interval (95% CI), mean abundance (MA) and mean intensity (MI) of the positive specimens per species. NC: not calculated.

	Fish species	No of specimens	No of positive specimens	P% (95% CI)	MI	MA
Large specimens	Black bullhead (*A. melas*)	77	22	28.6 (8.8–40)	1.7	0.49
	Wels catfish (*S. glanis*)	56	42	75.0 (61.6–85.6)	5.7	4.3
	Pumpkinseed (*L. gibbosus*)	23	1	4.3 (0.1–21.9)	1	0.04
Small specimens	Largemouth black bass (*M. salmoides*)	4	1	25 (6–80.6)	1	0.25
	Big-scale sand smelt (*A. boyeri*)	3500	180	5.1 (4.4–5.9)	1	0.05
	Small Thinlip grey mullet (*C. ramada*)	210	15	NC*	NC*	0.07

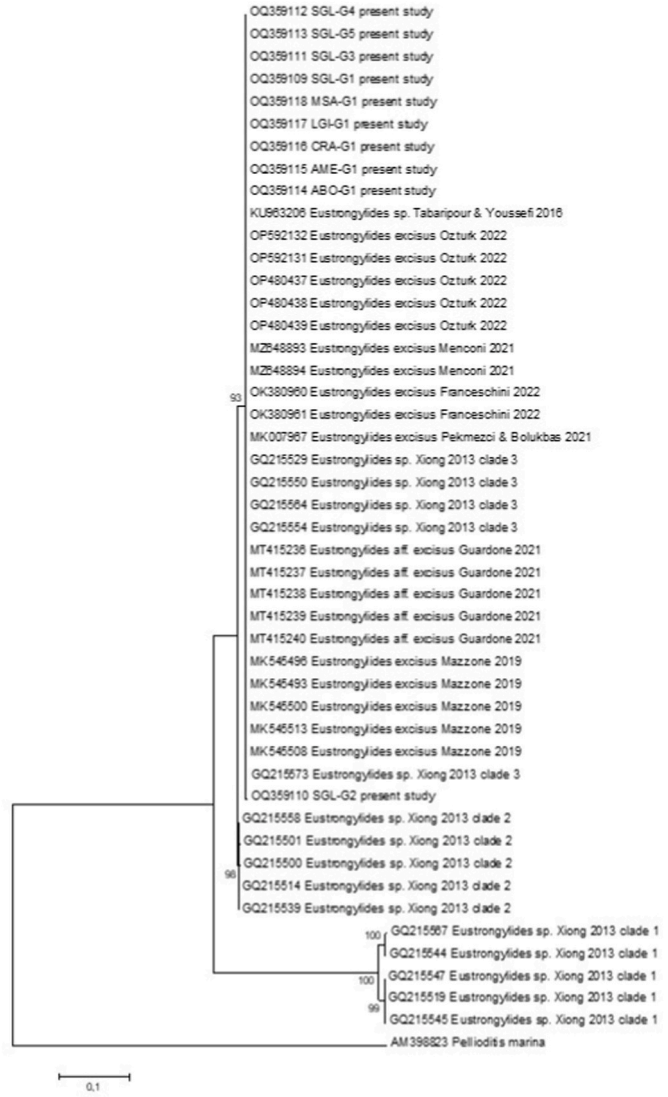

Fig. 3. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (−2179,7638) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Codon positions included were 1st+2nd+3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 584 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

et al. (2023).

The ML analysis showed a strong similarity of all the sequences produced in this study with those deposited by Mazzone et al. (2019), obtained from adult specimens of *E. excisus,* previously described morphologically. Thus, the species produced in the present study were therefore deposited as *E. aff. excisus*. Interestingly, the ten sequences produced in this study, and included in the phylogenetic analysis, clustered together with all the other sequences produced from *Eustrongylides* sp. larvae isolated from fish host collected from Italian lakes. The possibility of recognizing *Eustrongylides* species using sequencing and phylogenetic analysis is currently limited due to the lack of deposited sequences of *E. tubifex*, *E. mergorum* and *E. ignotus* produced from adult parasite specimens morphologically identified (Shamsi et al., 2023). However, our phylogenetic analysis supports the hypothesis that *E. excisus* is the only species present throughout Italian lakes. Finally, to build a solid molecular identification model, it is desirable to acquire adult nematodes specimens belonging to the different species through necropsy of definitive avian hosts and then to proceed to morphological classification in order to deposit reference sequences.

4.2. *The of Eustrongylides excisus expansion in Italy*

Reports on the presence of *Eustrongylides* spp. in fish from Italian lakes are recent. However, in less than ten years, after the first report in 2015 from the Trasimeno Lake (Dezfuli et al., 2015), a wide geographical distribution of this parasite (Fig. 4) and high prevalence in different fish species has been reported (Agnetti et al., 2016, 2019; Dezfuli et al., 2015; Franceschini et al., 2022; Guardone et al., 2021; Mazzone et al., 2019; Menconi et al., 2020; Rusconi et al., 2022). In addition, the number of fish species of commercial interest infected by this parasite has recently grown. In the study of Franceschini et al. (2022) the occurrence of the parasite was reported for the first time in three new host species:Black bullhead, Common carp and European eel.

The apparent expansion of this parasite in the lake areas of central and northern Italy is certainly a multifactorial phenomenon, but the population growth of one of its definitive hosts, the great cormorant, may have a major impact. In the mid-20th century the great cormorant population had reached its all-time low level in Italy, but in recent years it spread across the country and other areas of continental Europe, increasingly colonizing lakeside areas (Van Eerden et al., 2022; Volponi, 2005). The role of the cormorant in the recent geographical expansion of *Eustrongylides* spp. has been described in Measures (1988b), Mazzone et al. (2019), Rusconi et al. (2022) and Shamsi et al. (2023). Moreover, a correlation between the distribution of great cormorants, and in particular of their nesting activity, and the prevalence of *Eustrongylides* spp. in fish caught around nidification areas, has been demonstrated in Northwest Italy (Rusconi et al., 2022) and Southern Ukraine (Goncharov et al., 2018). In addition, as the cormorant population geographically expands, other avian species belonging to the order of Ciconiiformes, Pelecaniformes, and Anseriformes may also become infected, and possibly contribute to the further spread of the parasite (Measures, 1988b; Spalding & Forrester, 1993). Furthermore, water eutrophication, which results in the lowering of dissolved oxygen concentration, creates a more favorable environment for the survival of some aquatic oligochaetes, such as *Limnodrilus* sp., but also for a reduction of their predation. Both these factors lead to an increase in oligochaetes number, which is correlated with an increase in the presence of *Eustrongylides* spp. (Coyner et al., 2002; Hemaprasanth et al., 2020; Shamsi et al., 2023). Finally, also the rising temperatures may contribute to create a more favorable environment for the survival of *Eustrongylides* sp., which could also partly explain the reason for the increase in reports in Italy (Agnetti et al., 2019).

The water eutrophication has led to profound changes in the composition of the Massaciuccoli basin 's ichthyofauna over time. Zooplantophagous species, often not native to the lake such as Goldfish, Black bullhead, Wels catfish and Pumpkinseed, have been able to adapt their diets in response to environmental changes and have proliferated. In contrast, native species such as Pike (*Esox lucius*) and Tench, unable to compete with the others for resources, have almost disappeared. The results of our sampling are supported by previous studies describing the lake's ichthyofauna (Alessio et al., 1995; Macchioni et al., 2015). Indeed, the species that were most frequently collected during the entire sampling period were allochthonous and invasive species. Accordingly, Ercolini's study on Massaciuccoli basin's biodiversity (2015) asserts that the reduction of specimens belonging to native species in Lake Massaciuccoli is mainly caused by invasive species such as Black bullhead, Goldfish, and above all, by Wels catfish. Indeed, these were the three

Fig. 4. Geographical distribution of *Eustrongylides* spp. across in Italian lakes.

most collected species during the present study (Table 1).

4.3. Localization and prevalence of *E. excisus* larvae in different fish species from Lake Massaciuccoli

The infection level of every analyzed fish species was compared with literature data and interpreted considering their feeding habits and Massaciuccoli basin's ecology.

4.3.1. Large specimens

4.3.1.1. Positive species. Positive species are reported in Table 1. The parasitological analysis of the 56 Wels catfish specimens led to the detection of 241 *E. excisus* larvae, and to the calculation of the highest P value (P = 75%) found in the present study. The available data on Wels catfish report variable P values, ranging from 12% (Cirkovic et al., 2015) to 23% (Yardimci et al., 2018). Thus, the P recorded in this study is higher than those produced by other authors, except for the study by Abdybekova et al. (2020) in which all the 4 specimens analyzed were found positive.

The results of the parasitological analysis led to the calculation of a P value = 28.6% for the 77 Black bullhead specimens included in this study. However, considering that in the study of Franceschini et al. (2022) the P value (P = 2.72%) obtained for Black bullhead was calculated only by number of larvae found analyzing the belly flaps and not the viscera, it is not possible to compare the values. The same species was found to be negative in the study by Menconi et al. (2020), in which 30 specimens from Lake San Michele were analyzed. About the two catfish species, Wels catfish and Black bullhead, another relevant data was the MI of 5.73 and 1.72, respectively. These values can be linked to the dietary habits of those two species, which are generalist carnivores, and, in the case of the Wels catfish, also cannibalists. This leads them to feed on other intermediate hosts and to become in turn paratenic hosts for *Eustrongylides* spp. (Ercolini, 2015; Guardone et al., 2021; Leunda et al., 2008; Ruiz-Navarro et al., 2015; Vejřík et al., 2017). In this light, some species such as *S. glanis,* showing high value of MI could be used as an indicator (sentinel) to assess the presence of the nematode in freshwater ecosystems. When these species are not available, the choice of the target species should be made based on an aprioristic study of their feeding habits.

Among the 23 sampled Pumpkinseed, only one tested positive (P = 4.3%), with a larva found in the belly flaps. To date, four different reports of infected *L. gibbosus* are available for Italy, from Lake Garda (P: 3.7–12.5%, MI: 1), Lake San Michele (P: 18.3%, MI: 1.15) and Lake Trasimeno (P: 4.67–99.31%, MI: 1.27–2.57) (Franceschini et al., 2022; Menconi et al., 2020, 2021). The localization of the parasite, when reported, was visceral and/or muscular (Menconi et al., 2020, 2021).

As for the Largemouth black bass, the P value registered for this species (P = 25%) is to be considered of little significance because of the low number of specimens analyzed (n = 4).

The Pumpkinseed and the Largemouth black bass belong to the *Centrarchidae* family, together with the European perch (*P. fluviatilis*). For this latter species the correlation between the size and the level of infection was recently described by Franceschini et al. (2022). In fact, the reduction of P values in large specimens, could be justified by the diet change that occurs at some point of the fish growth. Indeed, while juveniles feed mostly on zooplankton and invertebrates (such as oligochaetes), adults feed on a wide variety of other fish species (Franceschini et al., 2022). This kind of diet change was also documented in Pumpkinseed and in Largemouth black bass and can lead to hypothesize a correlation between size and infection levels also in those two species (Copp et al., 2017; García-Berthou 2002; Ko et al., 2008).

4.3.1.2. Negative species. Parasitological analysis did not lead to any finding in five species (Table 1). For Goldfish, Common carp and Tench, all belonging to the *Ciprinidae* family, the negative results are in line with the literature. In fact, the mostly herbivorous and benthivorous feeding habits make those cyprinids not suitable as *Eustrongylides* spp. intermediate host (Lorenzoni et al., 2015; Macchioni et al., 2015; Manon & Hossain, 2011). On the contrary European eel is, from a dietary point of view, a generalist carnivore, that feeds mostly on zooplankton as juvenile and on other fish as adult, and for this reason suitable as

intermediate host for *Eustrongylides* spp. (Bouchereau et al., 2006; Ezzat & El-Seraffy, 1977; Golani et al., 1988). In the study conducted by Franceschini et al. (2022), this species tested positive, although with a low P (0.12–0.87%), found through belly flaps digestion. Other authors documented instead a much higher prevalence: P = 10–53% (Jakob et al., 2009) and P = 100% (Urdes et al., 2015). The reason why in the present study no positive European eel specimens were found is probably related to the fact that only few specimens were collected, presumably as a consequence of the current decline of the European eel population in the Massaciuccoli Lake (Ercolini, 2015).

All adults Thinlip grey mullet specimens tested negative while some larvae were found in juveniles. While adults are herbivorous, juveniles, inhabiting the so-called estuarine nursery areas, feed on benthic organisms, including oligochaetes (Tosi & Torricelli, 1988). Therefore, the same phenomenon described for the European perch could be hypothesized. However, currently no data for this species is available in literature, to support our results.

4.3.2. Small specimens

Big-scale sand smelt is one of the most investigated species in Italy for the presence of *Eustrongylides* spp. Our results agree with the literature. The parasitological exam of all the pools composed of Big-scale sand smelt specimens (total n = 3500 specimens), led to the finding of 180 *E. excisus* larvae. The mean P, considering all the P values of each pool (consisting of all the samples collected in a month), was equal to P = 5.1% (P range from 1.3 to 5.8%). Such values are in line with those obtained by Guardone et al. (2021) for the Massaciuccoli Lake (P = 2.3%). On the contrary, very variable P values were documented in Italy by Branciari et al. (2016) (P = 0.13–4%), Agnetti et al. (2019) (P = 69.5%) and Franceschini et al. (2022) (P = 0.06–40%). Çolak (2013), studying the presence of *Eustrongylides* spp. larvae in Big-scale sand smelt in Turkey, suggested that the variability in the infection prevalence could be related to a change in its feeding habits throughout the year; in fact, as an opportunistic and generalist carnivore, it exploits the available trophic resources, feeding in the cold months on benthic animals (such as infected oligochaetes) while in the warm months on plankton (Çolak, 2013).

Regarding the juvenile Thinlip grey mullets, two out of the three pools tested positive, but it was not possible to calculate a mean P, since an intensity >1 was occasionally detected (sa n. 1). The finding of 15 *E. excisus* larvae in this species sets a contrast with the fact that no adult individual tested positive.

Among small specimens, only stone moroko (Cyprinidae family) tested negative for *Eustrongylides* larvae, probably because this species feeds mainly on cyanobacteria and insects (Yalçin-Özdilek et al., 2013).

4.4. Implications for seafood quality and public health

Freshwater fishery products (fish, crustaceans, and cephalopods) represent only 5% of the EU production (EUMOFA, 2021). However, they may have a local commercial appeal (Pozio et al., 2013; Scaramozzino et al., 2018; Scholz et al., 2009) especially for the inhabitants close to lake areas, as well as for tourists, who could consume locally fished raw/undercooked fish dishes (Franceschini et al., 2022; Shamsi et al., 2023). Among the fish species collected in this study, the Big-scale sand smelt, together with eel, carp and pike were reported among the most appreciated species in the Massaciuccoli area (ARPAT, 2008). In particular, the Big scale sand smelt is widely consumed in Italy (Dal Bosco et al., 2019). As for catfish species (Black bullhead and Wels catfish), although their aquaculture production and consumption are common in East Europe (EUMOFA, 2021), these species, together with many other freshwater species, are also widely commercialized at the Milan wholesale fish market, the largest wholesale fish market in Italy (author's personal communication). Considering that in this study some species such as Black bullhead, Wels catfish, Pumpkinseed and Largemouth black bass, were found as hosts within the life cycle of *Eustrongylides* spp., as well as their tendency to accumulate large numbers of *Eustrongylides* spp., and their presence on the Italian market, the expansion of *Eustrongylides* spp. in Italian freshwater ecosystems and fish species must be considered in the light of possible public health and quality implications. With respect to the health implications, the genus *Eustrongylides* seems to be only responsible for rare cases of infections (Eiras et al., 2018). However, it has been recently hypothesized that cutaneous *Eustrongylides* may be of greater zoonotic importance than previously recognized (Williams et al., 2022). Moreover, in the light of the data demonstrating its ability to migrate both *intra-vitam* (Dezfuli et al., 2015) and *post mortem* (Agnetti et al., 2019) in the fish host, its impact on consumers health should be reconsidered. In particular, appropriate procedures for reducing its *post-mortem* migration, such as icing, evisceration and removal of belly flaps of landed fish should be applied. Regarding safety implications, the presence of *Eustrongylides* spp. larvae makes infected products unfit for human consumption (Regulation EC No 178/2002). In fact, *Eustrongylides* spp. larvae macroscopic appearance can cause the consumer's rejection with heavy impact on fish marketability (Franceschini et al., 2022). Therefore, despite the relatively low commercial interest of freshwater fish products, the establishment of specific risk management measures should be considered by FBOs. In fact, appropriate procedures (i.e., gutting and trimming) can be useful not only for assuring the safety of fishery products but also for guaranteeing their quality.

Regulatory requirements imposed by the EU establish risk management procedures for reducing the presence of visible parasites in the edible part of the fishery products. Regulation (EC) No 853/2004 states that "*food business operators must ensure that fishery products have been subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market*". Regulation (EC) No 2074/2005 establishes that "*visual inspection shall be performed on a representative number of samples*" during the gutting, filleting, and cutting of the fish fillets or fish slices, when these are carried out by FBOs. In this study the visual inspection was conducted according to the aforesaid Regulations during the parasitological examination (see section 2.1). Our results show that visual inspection allows to detect *Eustrongylides* spp. larvae both in large and small infected specimens, although with differences among species. In catfish (large specimens), 48% of the larvae (118 out of 247) found in Wels catfish and 39% of the larvae (15 out of the 38) found in Black bullhead, were detected through visual inspection. In small specimens, the number of larvae found by visual inspection was 62% (112 out of 180) in Big-scale sand smelt and 73% (11 out of 15) in juvenile Thinlip grey mullets (Table 1). The high proportion of larvae detected by visual inspection could be attributed to the bright red color and large dimension of *Eustrongylides* larvae that make them highly visible, especially in small specimens with a diaphanous appearance. In this light it could be interesting to assess possible discoloration of larvae after processing (cooking or pickling), considering that no studies are available to date.

Beside visual inspection, other procedures for preventing the migration of the larvae from the viscera and serosae to the edible muscular portion are icing and gutting. Even though no specific studies have been conducted on *post-mortem* migration of *Eustrongylides* spp. in fish, literature data is available for Anisakid nematodes (Cipriani et al., 2016). The Regulation (EC) No 853/2004 states that "*when 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*". Gutting is however feasible only in large specimens, while for small fishes, it is extremely time consuming for FBOs (Guardone et al., 2017). Interestingly, most of the larvae found in catfish specimens in this study were detected in the viscera and/or serosae and coelomic cavity: 88% (213 of the 241) of the larvae found in the Wels catfish specimens and 58% (22 of the 38) of the larvae found in Black bullhead specimens were in fact located at these levels (Table 1). Finally, together with gutting, one of the measures intended to reduce the parasite load in the edible tissues is the trimming of the belly flap. Even in this case this procedure is clearly

applicable only to large individuals that are intended for filleting. However, the trimming of the belly flaps can be considered an effective measure to reduce parasites load only when infection values are not excessively high. In this study we did not perform the enzymatic digestion of all the edible parts, that is considered the gold standard for detecting all parasite present (Guardone et al., 2016, 2018; Llarena-Reino et al., 2013). To propose a less time-consuming approach, feasible in self-control settings, we did not digest the axial muscles but only the belly flaps, as they represent a preferential location for nematodes larvae (Franceschini et al., 2022; Köse, 2010; Guardone et al., 2017). However, the cuts made on the axial muscles never led to the detection of any larvae. Therefore, even though we cannot exclude the presence of larvae in the edible part of the fish (such as axial muscles) we can assert that results obtained in this study could support the effectiveness of gutting and trimming procedures in lowering the overall parasite load in large size freshwater fish species.

As mentioned, gutting is too time-consuming to be systematically applied by FBOs for the detection of *Eustrongylides* spp. larvae in small fish species (Guardone et al., 2017). Therefore, in the Trasimeno Lake, to deal with the increasing presence of *Eustrongylides* spp. in small commercial fish species (Big scale sand smelt), the marketability of batches of small fish species is visually assessed only on a sample of 29 specimens for batches with more than 600 fish (Franceschini et al., 2022), as already applied for anchovies (Guardone et al., 2016). After the visual inspection an the number of visible larvae found is compared with an established threshold, and based on whether this threshold is exceeded, FBOs decide on the marketability of the entire batch (Franceschini et al., 2022). These measures could hypothetically also be undertaken by FBOs in the Massaciuccoli area to determine the suitability for consumption of batches of both big scale sand smelt and juvenile thinlip grey mullets. However, no preventive measures are currently applied because fishing and consumption of fish products from the Massaciuccoli lake are currently banned (Lucca Municipality Ordinance, 2011), issued due to the presence of microcystins (Bruno et al., 2009).

Action taken by FBOs to prevent the presence of *Eustrongylides* spp. larvae in freshwater fishery products are useful for reducing the likelihood of some larvae remaining in the edible portions of these products, but not to eliminate it altogether (EFSA, 2010). For this reason, it would be appropriate to educate consumers on proper handling and consumption, to reduce the parasite risk. In fact, the type of cooking can greatly influence the risk of human infection (Guardone et al., 2021). In all the human cases described so far raw products were always implicated (CDC, 1982; Eberhard, 1989; Narr, O'Donnell, Libster, Alessi, & Abraham, 1996). Specifically referring to thermic treatments, time-temperatures combinations prescribed by Regulation (EC) No 853/2004 to obtain the inactivation of parasitic nematodes (cooking at 60 °C at core for 10 min and freezing at −20 °C at core for 24 h), are supposed to be effective to kill *Eustrongylides* spp. larvae, although no specific studies on this topic have been conducted. Species such as Big scale sand smelt are widely consumed and used in various culinary preparations involving pan-frying or immersion-frying of whole fish (Dal Bosco et al., 2019), all preparations in which the temperatures reached are sufficient to provide the inactivation of the larvae. Even if no data is available on how juvenile Thinlip grey mullets are prepared and consumed, it can be assumed that, because of their similar size to the big scale sand smelt one, they are prepared and consumed in the same manner. For, Black bullhead and Wels catfish are species not normally used in typical Italian preparations, and it seems they are always cooked before consumption (author's note). A potential risk of parasite infection could occur in case of fraudulent substitution of species commonly consumed raw with catfish (Williams et al., 2020). A similar event was described for other freshwater species (Pozio et al., 2013).

Finally, no cases of allergic reactions following ingestion of fishery products contaminated with *Eustrongylides* spp. larvae have been reported, while this is documented for *Anisakis* spp. (Audicana et al., 2002; Daschner et al., 2012; Mattiucci et al., 2013). There is currently only a single study investigating the allergenic potential of *Eustrongylides* spp. larval antigens. In the study, the peritoneal inoculation of protein extract obtained from the nematode larvae in a murine model was associated to a rise in IgE antibodies (Kuraiem et al., 2021).

5. Conclusion

The present study contributed to deepen the knowledge on the occurrence of *Eustrongylides* spp. in fish species of the Massaciuccoli Lake (Northwest Tuscany), and more generally in Italian lakes. *Eustrongylides* spp. larvae were detected in *A. boyeri*, *M. salmoides*, *L. gibbosus*, *S. glanis* and *A. melas*, and for the first time in *C. ramada* juveniles. A possible correlation between the dietary habits of positive species and the infection level was proposed. Our molecular analysis, together with previous studies performed in Italy, support the hypothesis that *E. excisus* is the only species belonging to the *Eustrongylides* genus distributed throughout Italian lakes. In order to further characterize the larvae from a molecular point of view, it would be desirable to carry out the collection of adult nematodes from deceased great cormorants. The presence in the definitive hosts could also be suggestive of the parasite presence in the areas where cormorants usually nest. In a One Health perspective and to protect the consumers health, it is necessary to investigate all possible hosts of the parasite (birds, fish and invertebrate) to understand the reasons that lead to the presence of larvae in products intended for consumption. The possible presence of these species on the Italian market requires the application of risk management procedures by FBOs, which are especially necessary in large species such as Black bullhead, Wels catfish considering their tendency to accumulate large numbers of Eustrongylides spp. larvae.

CRediT authorship contribution statement

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

Declaration of competing interest

None

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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