Eustrongylides spp. parasite risk management in *Atherina boyeri* from Lake Trasimeno

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Abstract

Atherina boyeri is the primary source of fishing profit in Lake Trasimeno and a common host for *Eustrongylides* spp. larvae. The presence of *Eustrongylides* in fish is a public health concern, and effective risk management procedures are necessary to guarantee that infected products do not reach the market. Currently, in the European Union, there is no official sampling plan for fresh fish that defines sample size, inspection methods, and criteria for accepting or rejecting the product. An approach to *Eustrongylides* risk management is proposed in this study. A total of 270 batches of *A. boyeri*, each consisting of 29 specimens, were collected and examined visually in 3 years (2020-2023). The prevalence of the parasite was 20% in 2020, and in the first months of 2021, it grew up to ~40%, then dropped to 12.50% in December 2021 and settled at 16% in February 2022. In January and February 2023, the prevalence fell below 1%. The mean abundance was calculated and used to establish a threshold value to determine fish marketability. In 2020 and 2021, several batches were judged not marketable, and in some batches, a freezing treatment was implemented to ensure the inactivation of the parasite. In the last months of 2022 and in January and February of 2023, the presence of parasites in captured fish was negligible, and this allowed the marketability of fish as fresh. The proposed sampling plan was effective in preventing the commercialization of potentially hazardous products.

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Introduction

The global production in the aquaculture sectors and the consumption of fish are increasing, and the growing trend of eating raw or undercooked fish, such as sushi, sashimi, carpaccio, or fish tartare, has raised the risk of human exposure to the infected host of fish-borne parasites (Robertson, 2018). The primary causes of the increase in the incidence, geographic distribution, and frequency of zoonotic fish-related health issues are climate change and the rising demand for exotic, raw, and undercooked foods (Lõhmus and Björklund, 2015; Shamsi and Sheorey 2018). In light of some well-known parasitic zoonoses, researchers have recently focused on the propagation of *Eustrongylides*, a nematode found in many Italian and worldwide freshwater basins (Menconi *et al.,* 2020; Guardone *et al.,* 2021; Menconi *et al.,* 2021; Franceschini *et al.,* 2022; Morey *et al.*, 2022; Rusconi *et al.,* 2022; Honcharov *et al.,* 2022). Albeit few medical reports have been observed, *Eustrongylides* spp. has been identified as a zoonotic parasite that may represent a public health concern for consumers (Centers for Disease Control, 1982; Eberhard *et al.,* 1989; Wittner *et al.,* 1989; Narr *et al.*, 1996; Eberhard *et al.*, 2014).

The first parasite isolation in freshwater fish in Italy occurred in 2015 with perch (*Perca fluviatilis*, Linnaeus 1758) from Lake Trasimeno (Umbria, Italy) (Dezfuli *et al.,* 2015). Its existence in the same lake was later observed in other commercially important fish species (Branciari *et al.*, 2016; Franceschini *et al.*, 2022). The parasite has recently been found in fish from various lakes in northern Italy and two lakes in central Italy (Table 1) (Dezfuli *et al.*, 2015; Branciari *et al.,* 2016; Agnetti *et al.,* 2019; Covelli J, 2019; Mazzone *et al.,* 2019; Parco Valle Lambro, 2019; Menconi *et al.*, 2020; Guardone *et al.*, 2021; Menconi *et al.*, 2021; Franceschini *et al.,* 2022; Rusconi *et al.*, 2022; Castiglione *et al.*, 2023).

In freshwater ecosystems, nematodes of the genus *Eustrongylides* (family *Dioctophymatidae*) have a complex indirect life cycle characterized by 5 stages, with fish-eating birds serving as definitive hosts (Measures, 1988; Spalding *et al.,* 1993; Spalding and Forrester, 2008). The first intermediate hosts are aquatic oligochaete worms, *Lumbriculus variegatus*, *Tubifex,* or *Limnodrilus* spp., which ingest parasite eggs shed through birds' feces. Inside the oligochaete, the parasite develops into the second and third larval stages (Bjelić-Čabrilo *et al.*, 2013). The second intermediate host is planktivorous and benthivorous fish, in which the third-stage larvae transform and molt into the fourth larval stage and remain in the fish (Spalding and Forrester, 2008; Cole, 2009). Predatory fish, as well as amphibian and reptile species, can serve as paratenic or transport hosts (EFSA, 2007; Bjelić-Čabrilo *et al.*, 2013), and they are capable of infecting birds or (accidentally) humans through the consumption of raw or undercooked freshwater fish.

Research conducted in Italy identified all collected *Eustrongylides* spp. as *E. excisus*, one of the 3 species already characterized (together with *E. tubifex* and *E. ignotus*) (Measures, 1988).

Given the wide spread of *Eustrongylides* spp. in Italian lakes, the only prevention method is the use of an effective control system by trained food business operators (FBOs), as settled by the legislation. Indeed, the European Commission established that FBOs must ensure that fishery products have been subjected to a visual examination to detect visible parasites before being placed on the market (European Commission, 2005). The study of its presence in Italian lakes helps understand its epidemiology as well as the need for appropriate monitoring and sanitary measures (Agnetti *et al.*, 2016), especially given the increased consumer and media attention to parasites in freshwater fish.

Different methods for detecting visible parasites in fish have been proposed, including visual inspection, which must be performed on a representative number of samples (European Commission, 2005). Nevertheless, there are no legal requirements in Europe or Italy that define a detailed sampling plan to be used for the collection of fresh fish to be visually inspected (European Commission, 2005). A sampling plan, by definition, includes the sample size, the inspection procedure, and the criteria to be used to accept or reject the batch of production based on the inspection results (Codex Alimentarius Commission, 1969).

In Lake Trasimeno, *Atherina boyeri* (Risso, 1810 - big-scale sand smelt) is the dominant species of the fish community. This species is a small coastal fish living in the Mediterranean and Northeast Atlantic Sea basins (Quignard and Pras, 1986; Whitehead *et al.,* 1986; Bianco *et al.,* 2013). *A. boyeri* is widespread in coastal and estuarine waters and lagoons with a wide range of salinities (from freshwater to hypersaline), but it is also found on occasion in freshwaters. Numerous consistent freshwater resident populations have also been observed in Lake Trichonis in Greece and the Iberian Peninsula's Santo André lagoon (Freyhof and Kottelat, 2008). Besides that, this euryhaline species has been successfully introduced into many lakes, either intentionally or accidentally (Freyhof and Kottelat, 2008). Although the fish is native to Italy, introductions into numerous lakes have allowed it to spread far beyond its natural habitat (Bianco *et al.,* 2013). The big-scale sand smelt was most likely accidentally introduced into Lake Trasimeno in 1920, along with juveniles of other commercially important species (Lorenzoni *et al.,* 2015). *A. boyeri* was first caught by

Table 1. Presence of *Eustrongylides* in Italian freshwater basins.

	Lake	Parasitized fish species/host	References
Lombardy	Ceresio	Perch (*Perca fluviatilis*)	Covelli, 2019
	Montorfano	Common Goby (*Padogobius martensii*)	Parco Valle Lambro, 2019
	Annone	Perch (*Perca fluviatilis*)	Rusconi *et al*., 2022
Veneto	Garda	Perch (*Perca fluviatilis*) Largemouth black bass (*Micropterus salmonids*) Pumpkinseed sunfish (*Lepomis gibbosus*)	Menconi *et al*., 2021
Piedmont	San Michele	Perch (*Perca fluviatilis*) Largemouth black bass (*Micropterus salmonids*) Pumpkinseed sunfish (*Lepomis gibbosus*)	Menconi *et al*., 2020
Umbria	Trasimeno	Perch (*Perca fluviatilis*)	Dezfuli *et al*., 2015 Mazzone *et al*., 2019 Branciari *et al*., 2016 Franceschini *et al*., 2022
		Largemouth black bass (*Micropterus salmonids*)	Branciari et al., 2016 Franceschini *et al*., 2022
		Big-scale sand smelt (*Atherina boyeri*)	Branciari *et al*., 2016 Franceschini *et al*., 2022 Agnetti *et al*., 2019
		Eel (*Anguilla anguilla*) Carp (C*yprinus carpio*) Black bullhead (*Ameiurius melas*) Pumpkinseed sunfish (*Lepomis gibbosus*) Tench (*Tinca tinca*)	Franceschini *et al*., 2022
Tuscany	Massacciuccoli	Big-scale sand smelt (*Atherina boyeri*)	Guardone *et al*., 2021 Castiglione *et al*., 2023
		Black bullhead (*Ameiurus melas*) Small Thinlip grey mullet (*Chelon ramada*) Wels catfish (*Silurus glanis*)	Castiglione *et al*., 2023

commercial fishermen in 1928 (Lorenzoni *et al.*, 2015). Before 1980, big-scale sand smelt was mainly irrelevant to the fish caught, but in the years since, it has emerged as a significant source for the commercial catches of local fishermen, accounting for 50% of the total catch, as previously mentioned (Lorenzoni and Ghetti, 2012).

This work reports the result of a visual inspection performed under Regulation 2074/2005 on *A. boyeri* specimens collected at the *Cooperativa Pescatori del Trasimeno*, according to an implemented sampling plan proposed by the FBOs. The proposed sampling plan was used to define the fish marketability. Another objective of the study was to examine the frequency of *Eustrongylides* in *A. boyeri* over 3 years (2020-2023) to support emerging concerns about the presence of *Eustrongylides* in this fish product, whose consumption is rising because it is a primary source of commercial catches for nearby fishermen.

Materials and Methods

Food business operators of the freshwater fish supply chain

This study was carried out in collaboration with FBOs (*Cooperativa Pescatori del Trasimeno*), which are represented by the fishermen's cooperative in the municipality of San Feliciano, Perugia, Italy. Each member of the cooperative has a fishing boat, and they work along the east shore of Lake Trasimeno, which is the fourth-largest laminar lake in Italy (about 128 km^2) and is situated in the Umbrian region at 43°08'N and 12°06'E (Branciari *et al.*, 2017). It is quite shallow, with an average depth of 4.72 m and a maximum depth of 6.3 m and it is a closed lake with no natural outlets. These characteristics cause the lake's water level to vary significantly with the amount of local rainfall, making weather and climate the only real determinants of the lake's water level (Gravina *et al.,* 2022). The lake is considered a high-value ecosystem due to its wide biodiversity; indeed, its fish fauna comprises 19 species, dominated by those belonging to the *Cyprinidae* family (Branciari *et al*., 2020). Nowadays, *A. boyeri* is the dominant species in Lake Trasimeno's fish community, and it is the primary source of fishing profits (Lorenzoni *et al.,* 2015).

Sample collection

Specimens of big-scale sand smelt were caught by professional fishermen of Lake Trasimeno from January 2020 to January 2023 (during the fishing season, from January to March and from October to December). Fish were caught using pots of the size of 6 mm placed in 3 different locations on the east shore of Lake Trasimeno (Figure 1). The locations were chosen by the fishermen because they were clear of submerged hydrophytes and therefore more frequented by the species. The caught fish is disembarked within 2 hours and immediately transferred to European Union-approved reception plants, where fishes are boxed, weighed, covered with ice, and moved to a temperature stabilization room.

Visual inspection procedure and fish marketability

To avoid placing "obviously contaminated products" on the market, European legislation requires all FBOs to conduct a non-destructive visual inspection of fishery products for the detection of "visible parasites, which in terms of size, color, or texture is distinguishable in fish tissues" (European Commission, 2005). The same Regulation states that "a representative number of samples shall be visually inspected during production. The scale and frequency of inspections shall be determined by the persons in charge of establishments based on the type of fishery products, their geographical origin, and their use" (European Commission, 2005). As a result, FBOs are urged to include correct preventive procedures in their self-control plans for managing the risk of parasites in fishery products and discarding heavily infected fish, which can have a direct impact on product quality and consumer health. Consequently, a modified sampling plan for sand smelt was developed in accordance with Franceschini *et al.* (2022), based on a procedure proposed for managing *Anisakis* risk in anchovies (Lombardy Region, 1994; Liguria Region, 1997; Guardone *et al.,* 2017). Based on the inspection results, the plan specified the sample size as well as the criteria for accepting or rejecting the batch of production. The total weight of the fish lot was required to calculate the total number of specimens and, consequently, the number of subjects to be inspected.

The number of specimens for each batch was determined using Epitools-Epidemiological Calculators (Ausvet, Fremantle, Australia), an online platform with several functions such as estimating sample size and disease prevalence. 217 batches, each composed of 29 subjects, were examined over 3 years. The veterinary consultant for *Cooperativa Pescatori del Trasimeno* carried out fish sampling and visual inspection as follows: observation, in good lighting conditions, of the nematode from the outside of the fish; then, due to the fish's transparency, the ventral surface of each fish was opened longitudinally to examine the viscera and visceral cavity under good lighting conditions. Because of the small size of the fish, the procedure allowed for a complete opening of each specimen. Following a visual inspection, the larvae in each batch were counted to determine the mean abundance (MA), which was used to establish a cut-off point for fish marketability.

Mean abundance

MA was calculated following the criteria of Bush *et al.* (1997), considering the total number of individuals of a specific parasite species in a sample of a specific host species divided by the total number of hosts of that species examined. MA was used to define the criteria to accept or reject the batch based on the results of the sample examination. The visual inspection verifies the presence of larvae (considering the size of the fish, each fish can only be parasitized by a larva, mean intensity equal to 1), and the definition

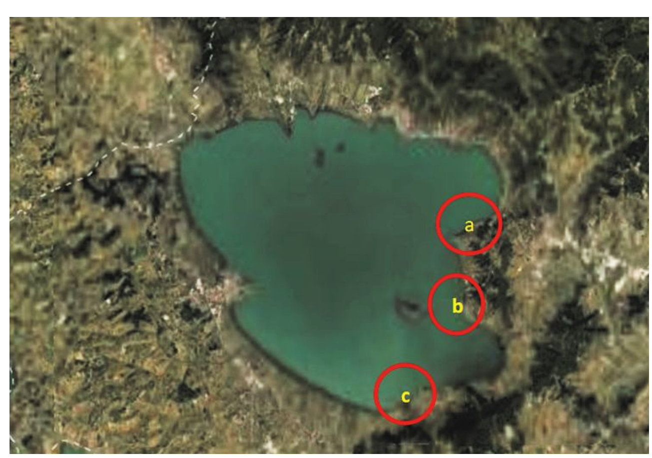

Figure 1. Landing points of the catch. **a**) Torricella; **b**) San Feliciano; **c**) Sant'Arcangelo.

of fish marketability is as follows: i) if the number of parasitized fish is higher than 10% of the total examined, the batch should be submitted to decontamination by means of freezing (-20°C for 24 hours); ii) if the sampled specimens have massive infestation, with a repulsive appearance, the batch should be discharged; iii) if the number of larvae is < in maximum 10 % of the examined specimens, the batch is intended to free consumption. MA was used to establish a threshold for the marketability of the batch which means that an MA of 0.1 (3 fish, corresponding to a total of 3 larvae in 29 specimens) corresponds to the threshold that allows dividing the batches into marketable (MA≤0.1) or non-marketable (marketable after freezing or never marketable; MA>0.1).

Statistical analysis

The prevalence and 95% confidence intervals (CI) of *Eustrongylides* spp. were estimated in fish species by an exact method based on the binomial distribution. The prevalence, 95% CI, and MA were calculated according to Bush *et al.* (1997).

Results and Discussion

Figure 2 presents the visual inspection of *A. boyeri* performed on 29 specimens for batch. One of the locations where the larvae were discovered on the host body was the skin's epithelial layer (Figure 3). In some cases, the larva was located in the coelomic cavity. On the contrary, Guardone *et al.* (2021) reported that in infested sand smelt caught in Lake Massaciuccoli (Tuscany), the majority of the larvae were located in the viscera and muscle. Furthermore, Guardone *et al.* (2021) reported that in a few specimens, the fish's transparency, its small size, and the characteristics of the larvae allowed for the observation of the nematode from the outside of the fish (Figure 3).

Regulation 853/2004 enforced that FBOs ensure that fishery products are subjected to a visual examination for the detection of visible parasites before being placed on the market (European Commission, 2004). To ensure food safety, FBOs should implement appropriate sampling plans and analytical methods prescribed by European or national legislation. In the absence of such protocols, scientifically validated alternatives should be used (European Commission, 2004; Guardone *et al.,* 2017). A procedure was proposed by FBOs for managing *Eustrongylides* risk in big-scale sand smelt (Franceschini *et al.,* 2022). The plan first established the sample size for each batch (daily capture), considering a 10% sensitivity; this meant that there would be 29 subjects for analysis. The results of the 217 batches examined, prevalence, MA and threshold value of *Eustrongylides* spp. in sand smelt are reported in Table 2.

The acceptable batches were those with a number of parasitized specimens up to 10% of the sample analyzed [3 fish, corresponding to a total of 3 larvae (Guardone *et al.,* 2017)], giving an MA threshold of 0.1. In the last two months of 2020 and the first two months of 2021, several batches were judged unfit for human consumption and not marketable, causing a temporary stop in fishing this species and a consequent loss of income for the fishermen. In the last three months of 2021 and the first months of 2022, the number of parasitized specimens often required the application of a freezing treatment to ensure the inactivation of parasites. After the summer of 2022, the presence of parasites in captured fish was negligible, and the criteria established allowed for the marketability of fresh sand smelt caught in 2022 and 2023.

A high prevalence was recorded at the end of 2020 (20%) and in the first months of 2021 (up to ∼40%), then the prevalence dropped to 12.50% in December 2021 and settled at 16% in February 2022. The prevalence in the last months of 2022 dropped to values below 2%. In January and February 2023, the prevalence settled at a value below 1%.

Few studies are available regarding the presence of *Eustrongylides* spp. in big-scale sand smelt. Guardone *et al.,* (2021) reported the presence of the nematode in *A. boyeri* Lake Massaciuccoli with a prevalence of 2 %; in Europe, only Çolak (2013) found *Eustrongylides excisus* larvae in sand smelt caught in Lake Iznik, Turkey. Çolak (2013) also found a higher prevalence of *Eustrongylides* during the winter season, and a possible reason is that sand smelt feeds on zoobenthos, potentially including the infected oligochaete. Moreover, Çolak (2013) studied the feeding habits of sand smelts and discovered that this species is an

Figure 2. Visual inspection of 29 specimens of sand smelt: **a**) whole specimens; **b**) specimens after visual inspection (viscera observation).

Figure 3. Skin epithelial *Eustrongylides* spp. locations in sand smelt.

Table 2. The number of batches examined in 3 years (2020-2023) during fishing season, and the results of visual inspection, mean abundance, and prevalence.

Year	Months	Batch number	Specimens examined	Number of larvae	MA	Batches discharged	p% (95 CI)
2020	January	10	290	29	0.10	0	10.00 (6.54-13.45)
	February	11	319	22	0.07	0	6.90 (4.11-9.67)
	March	13	377	39	0.10	0	10.34 (7.27-13.41)
	October	13	377	104	0.28	0	27.58 (23.07-32.09)
	November	13	377	137	0.36	7	36.34 (31.48-41.19)
	December	9	261	90	0.34	3	34.48 (28.71-40.24)
2021	January	20	580	231	0.40	13	39.82 (35.84-43.81)
	February	15	435	165	0.38	9	37.93 (33.37-42.49)
	October	16	464	107	0.23	1	23.06 (19.22-26.89)
	November	16	464	58	0.13	0	12.50 (9.49-15.51)
	December	16	464	58	0.13	0	12.50 (9.49-15.51)
2022	January	16	464	65	0.14	0	14.01 (10.85-17.16)
	February	2	58	9	0.16	0	15.51 (6.19-24.83)
	October	5	145	10	0.07	0	6.90 (2.77-11.02)
	November	20	580	18	0.03	0	3.10 (1.69-4.51)
	December	15	435	7	0.02	0	1.60 (0.42-2.79)
2023	January	4	116	1	0.01	0	0.86 (0-2.54)
	February	3	87	0	0.00	0	0 (0.00-0.00)

MA, mean abundance; CI, confidence interval.

opportunistic carnivore that feeds on both planktonic and benthonic prey at various times of the year. The same feeding habits were also confirmed by Guardone *et al.,* (2021) in Lake Massaciuccoli where this species is known to feed on zooplankton and zoobenthos, including oligochaetes. The prevalence found in this study for *A. boyeri* in the first months of 2023 is similar to that registered in Lake Trasimeno in 2016.

The parasite's consistent presence in Lake Trasimeno over the years, and specifically the presence of infested sand melt, could be attributed to the growth of the cormorant (*Phalacrocorax carbo*) population, which is concentrated around the lake, most likely due to changing migratory patterns caused by climate change, as well as the possibility that environmental factors (nutrients, organic compounds, temperature, *etc*.) favor oligochaete population growth, as confirmed by Goncharov *et al.* (2018). Nevertheless, despite an increasing prevalence rate starting from 2016 registered in sand smelt up to 2021 (Franceschini *et al.,* 2022), in the last two years, the prevalence of infested hosts has decreased remarkably even though the cormorant population has remained stable or even increased (Table 1) (Dezfuli *et al.*, 2015; Branciari *et al.,* 2016; Agnetti *et al.,* 2019; Covelli, 2019; Mazzone *et al.,* 2019; Parco Valle Lambro, 2019; Menconi *et al.*, 2020; Guardone *et al.*, 2021; Menconi *et al.*, 2021; Franceschini *et al.,* 2022; Rusconi *et al.*, 2022; Castiglione *et al.*, 2023). Generally, eutrophication and warm water temperatures create optimal conditions for the parasite. Water quality is an important factor that, in some situations, is subject to actions that may decrease transmission of the parasite (Pietrock and Marcogliese, 2003). Regarding *Eustrongylides* spp., no parasite reduction has been reported in the literature; nevertheless, an explanation of this singular situation could probably be attributable to climate change and the extreme temperature of the shallow lake water during summer. Extreme water temperatures most likely impacted the survival of the parasite cycle's intermediate hosts, reducing the likelihood of the parasite spreading.

Conclusions

The current study describes the visual inspection procedure used in a freshwater fish processing plant. Due to the parasite's widespread distribution in Lake Trasimeno, a suitable self-control strategy is needed to guarantee its eradication or reduction in the final product. Even though the presence of *Eustrongylides* larvae in fishery products poses a limited threat to public health, it leads to a deterioration in the quality of the product, making it repugnant to consumers. As a result, a risk management procedure is required to prevent products containing viable larvae from reaching consumers, but it is also appropriate to implement corrective measures to reduce consumers' perception of the issue, as occurred recently. Moreover, FBO self-checks for *Eustrongylides* risk management help provide epidemiological data on the parasite's spread.

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