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Research Note

Prevalence of *Anisakis* spp. and *Hysterothylacium* spp. Larvae in Teleosts and Cephalopods Sampled from Waters off Sardinia

GIULIA ANGELUCCI,[1] MAURO MELONI,[1] PAOLO MERELLA,[2] FRANCESCO SARDU,[3] SALVATORE MADEDDU,[4] RAFFAELE MARROSU,[5] FRANCO PETZA,[4] AND FULVIO SALATI[1]*

[1]*Fish Disease and Aquaculture Center, IZS of Sardinia, State Veterinary Institute, Via Parigi s.n., 09170 Oristano, Italy;* [2]*Sezione di Parassitologia e Malattie Parassitarie, Dipartimento di Biologia Animale, Università di Sassari, 07100 Sassari, Italy;* [3]*Azienda Sanitaria Locale ASL 5 (Local Sanitary Authority), 09170 Oristano, Italy;* [4]*Azienda Sanitaria Locale ASL 8 (Local Sanitary Authority), 09100 Cagliari, Italy; and* [5]*Azienda Sanitaria Locale ASL 1 (Local Sanitary Authority), 07100 Sassari, Italy*

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ABSTRACT

A study was carried out on the presence of *Anisakis* and *Hysterothylacium* larvae in fish and cephalopods caught in Sardinian waters. A total of 369 specimens of 24 different species of teleosts and 5 species of cephalopods were collected from different fishing areas of Sardinia. Larvae were detected and isolated by both visual inspection and enzymatic digestion. These methods allowed *Anisakis* type I and type II third-stage larvae and *Hysterothylacium* third- and fourth-stage larvae to be detected. The prevalence, mean intensity, and mean abundance were calculated. The results obtained showed the highest prevalence of Anisakidae in *Zeus faber* (100%) and of *Anisakis* in *Micromesistius poutassou* (87.5%). The highest prevalence of *Anisakis* type I larvae was in *M. poutassou* (81.2%), and that of *Anisakis* type II larvae was in *Todarodes sagittatus* (20%). The highest values for prevalence, mean intensity, and mean abundance for *Hysterothylacium* were found in *Z. faber*. These prevalences and the mean intensity and abundance were higher than those reported by different authors in other Mediterranean areas. This may be because the enzymatic digestive method used in this research resulted in higher recovery levels. The data suggest that Sardinia may be a high-risk area for zoonotic diseases and that measures such as information campaigns, aimed at both sanitary service personnel and consumers, should be employed to limit the spread of such zoonosis.

Anisakids are parasitic aquatic nematodes whose life cycles involve crustaceans, cephalopods, fish, marine mammals, and sea birds. These nematodes are important because they cause zoonosis *(55)*. The accidental intake of these parasites can cause digestive disorders and/or allergies in humans *(5, 7, 14, 40, 44, 45)*. This generally occurs through the consumption of raw or poorly cooked infected fish *(7, 14, 40)*. *Anisakis* is the most important and most intensively studied anisakid nematode. It is also the anisakid nematode that is most frequently involved in cases of human seafood infections. This is because viable larvae can penetrate the human gut and cause gastrointestinal syndromes *(1)*.

In recent decades there has been an increase in the consumption of raw fish dishes, such as Japanese sushi and sashimi, in Europe and the Mediterranean area. However, it is not only these dishes that are potentially hazardous, but also traditional ones such as Dutch salted or smoked herring, Nordic gravlax (dry, cured salmon), Spanish *boquerones en vinagre* (pickled anchovies), and Italian *acciughe marinate* (marinated anchovies) *(4, 44)*. Anchovies in particular are known to have the highest prevalence (P) of *Anisakis* among fish species, and they are very commonly used in certain raw fish dishes in the Mediterranean area *(46)*. Most Spanish cases of anisakiasis have indeed been blamed on the consumption of *boquerones en vinagre,* and in Italy consumption of *acciughe marinate* is the main cause of this disease *(8, 15, 16, 22, 25, 32, 33, 35, 47)*. This increasingly widespread combination of old and new culinary habits has meant that the probability of anisakids being ingested has increased, even in areas where raw fish was not part of the traditional diet.

In the last 10 years, the National Health and Sanitary Services of Europe and Italy have reported about 100 cases of seafood poisoning where the symptoms were not dissimilar to acute or chronic anisakiasis. However, anisakiasis symptoms are not specific, and so the disease is not always diagnosed immediately. At the moment there is no specific medical treatment that kills the parasites in vivo *(4, 21, 33, 36, 43–45, 51)*.

At present, the health authorities and the fish industry are aware of the problem, which can affect both human health and the commercial value of the product. There are strict hygienic controls of fish products destined for processing or direct human consumption. However, sanitary inspection by highly qualified personnel at wholesale and retail level does not ensure that the product is completely safe. There are objective difficulties, such as the use of random sampling (Regulations EC No 853/2004, EC

* Author for correspondence. Tel: +39-328-6256492; Fax: +39-(0)783-358931; E-mail: fulviosal@hotmail.com.

No 854/2004, and EC No 2074/2005) *(4, 17–19)*. The preparation of fish at home or the incorrect storage of fresh products in restaurants also increases the risk of infection. These nematodes are frequently found in wild fish, and checking how they are treated after they are caught and during processing (i.e., evisceration, freezing before transformation, and random inspection by veterinarians) is fundamental for the prevention of human parasitosis, but these inspections are not always completely effective. The larvae can also move from one paratenic host to another in the food chain, thus increasing the number of nematodes in larger and older organisms *(49)*. This can have important effects on both epidemiology and food safety in general.

Despite the commercial and zoonotic importance of anisakid infections, the distribution and epidemiology of this nematode in the Mediterranean Sea are little studied, especially when compared with the surveys carried out in the Atlantic Ocean *(2, 10, 28, 38, 50)*. In addition, some authors study the presence and P of anisakid larvae in a particular Mediterranean teleost species only *(3, 23, 38, 46, 52–54)*. Thus, it is necessary to define the epidemiological situation of anisakid larvae in the Mediterranean (e.g., along Sardinian coasts), as well as to investigate the location (i.e., body cavity or muscles) and/or migration of the larvae after the fish is caught. Epidemiological data on *Anisakis* diffusion are also very important from a legal point of view *(17, 19)*. Moreover, it is important to know whether the worms localize in muscular tissues of the paratenic host (teleost or cephalopod), because humans are infected by anisakid larvae after the ingestion of poorly cooked or raw fish.

The aim of this work was to study the presence and prevalence of Anisakidae, and particularly *Anisakis* and *Hysterothylacium,* in different species of wild fish and cephalopods from the coastal waters and lagoons of Sardinia.

MATERIALS AND METHODS

Samples. A total of 369 specimens of teleosts (24 species) and cephalopods (5 species), were collected from different sites off the coasts and in the lagoons of Sardinia between October 2008 and January 2010. The uneviscerated samples were delivered to the laboratory in coolers kept at +4°C within 12 h after fishing and were immediately examined to test for the presence of anisakid larvae in the body cavity.

Inspection. The inspection of the hosts consisted of (i) identifying the fish or cephalopod genus and species following the ''Fiches FAO d'identification des espèces pour les besoins de la pêche'' *(24)* (in particular, the genus *Trachurus* included *T. trachurus, T. mediterraneus,* and *T. picturatus*; the genus *Mullus* included *M. barbatus* and *M. surmuletus*; the genus *Diplodus* included *D. sargus sargus, D. puntazzo, D. annularis,* and *D. vulgaris*; the genus *Spicara* included *S. maena* and *S. flexuosa*; and the genus *Octopus* included *O. vulgaris* and *O. macropus*); (ii) recording the length and weight of the host; (iii) opening the body cavity for visual inspection and searching for anisakid larvae with an illuminated magnifying glass; (iv) washing the body cavity with distilled water and collecting the parasites on a petri dish; (v) counting the anisakid larvae found; (vi) identifying the worms collected as *Hysterothylacium* spp. or *Anisakis* spp. type I or II larvae, sensu Berland, at the genus level with an optical microscope (4× or 10× lens objective) *(6)*; and (vii) storing the host and its viscera separately at −20°C until the digestion process. When the larvae could not be identified microscopically by the above method, they were fixed and mounted on a slide with 70% ethanol and a few drops of glycerol and then observed through an optical microscope (4×, 10×, or 20× lens objective).

Enzymatic digestion. Enzymatic digestion was performed on samples previously stored at −20°C. The viscera and muscles were digested separately in order to establish whether *Anisakis* was present at the muscular level. The enzymatic digestion method used was based on the *Codex Alimentarius* and the EU Regulation No 2075/2005 *(11, 20)*.

In brief, the slightly modified method was as follows: 100 g of viscera or muscles of each sample was put into a beaker containing 10 g of pepsin powder (Sigma-Aldrich, St. Louis, MO), 16 ml of HCl (37%) (Carlo Erba, Rome, Italy), and 2 liters of distilled water. The mixture was heated at 37°C and continuously stirred for 1 or 2 h, depending on whether a fish or cephalopod, respectively, was being digested. It was then filtered through two sieves with different mesh sizes (1 and 0.5 mm). The sieves were rinsed with distilled water, and the collected material was observed through a stereomicroscope.

Data processing. The data obtained by inspection and digestion were processed as follows in order to calculate the prevalence (P), mean intensity (MI), and mean abundance (MA): P = number of infected hosts/total number of examined hosts × 100; MI = number of parasites/number of infected hosts; MA = number of parasites/total number of examined hosts.

RESULTS AND DISCUSSION

Table 1 shows the P values of Anisakidae and *Anisakis* spp. larvae found in teleosts and cephalopods. The total P of Anisakidae was 62.3%, whereas that of *Anisakis* spp. was 24.4%. The highest P values of Anisakidae larvae were found in *Zeus faber* (100%), *Trachurus* spp. (96.6%), and *Micromesistius poutassou* (93.7%). The highest P values of *Anisakis* spp. were slightly different: *M. poutassou* (87.5%), *Scomber scombrus* (55.6%), and *Trachurus* spp. (52.5%). The same table shows the P, MI, and MA of *Anisakis* spp. (type I and type II) and *Hysterothylacium* spp. among the different species of teleosts and cephalopods. The hosts had a total P of 23.8% of *Anisakis* type I larvae, whereas the figures for MI and MA were 8.09 and 1.93, respectively. The highest values for P of *Anisakis* type I were found in *M. poutassou* (81.2%), in *S. scombrus* (55.6%), and in *Trachurus* spp. (52.5%). The data for *Anisakis* type II larvae were 1.35% for P, 1.80 for MI, and 0.02 for MA of the total examined hosts. This type of larva was found only in four host species (P values given in parentheses): *Todarodes sagittatus* (20.0%), *M. poutassou* (12.5%), *Z. faber* (10.0%), and *Trachurus* spp. (1.7%).

Of the 369 examined hosts, 174 were infected by *Hysterothylacium* spp. (P, 47.2%) with an MI of 17.2 and an MA of 8.13. The highest values were in *Z. faber* (P, 100%), *Trachurus* spp. (P, 77.9%), and *Mullus* spp. (P, 73.0%) (Table 1).

The presence of *Anisakis* larvae in muscle is shown in Table 2. The total P was 12.7%, while total MI was 1.06. *Anisakis* spp. larvae located in the muscle and isolated by enzymatic digestion were found in only five fish species, in

TABLE 1. *Prevalence, mean intensity, mean abundance, and number of larvae of Anisakidae,* Anisakis *spp.,* Anisakis *type I and type II, and* Hysterothylacium *spp. in the sampled teleosts and cephalopods*[a]

Species	No.	No. of specimens infected with:					P (%)					No. of larvae			MI			MA		
		AN	An. spp.	An I	An II	Hyst	AN	An. spp.	An I	An II	Hyst	An I	An II	Hyst	An I	An II	Hyst	An I	An II	Hyst
Trachurus spp.	59	57	31	31	1	46	96.6	52.5	52.5	1.7	77.9	437	1	1,652	14.1	1	35.9	7.4	0.02	28
Engraulis encrasicolus	52	40	18	18		35	77.0	34.6	34.6		67.3	65		125	3.6		3.6	1.25		2.4
Merluccius merluccius	50	31	15	15		21	62.0	30.0	30.0		42.0	25		122	1.7		5.8	0.5		2.44
Mullus spp.	48	38	3	3	3	35	79.0	6.2	6.2		73.0	3		389	1		11.1	0.06		8.1
Octopus spp.	26	1				1	3.8				3.8			4			4			0.15
Liza ramada	22	3					13.6													
Micromesistius poutassou	16	15	14	13	2	8	93.7	87.5	81.2	12.5	50.0	130	6	31	10	3	3.9	8.12	0.37	1.94
Diplodus spp.	15	3				2	20.0				13.3			3			1.5			0.2
Sparus aurata	10	2					20.0													
Zeus faber	10	1		1	1	10	100	10.0	10.0	10.0	100	29	1	468	29	1	46.8	2.9	0.1	46.8
Scomber scombrus	9	7	5	5		2	77.8	55.6	55.6		22.2	21		2	4.2		1	2.33		0.22
Lithognatus mormyrus	6	2				1	33.3[b]				16.7			1			1			0.17
Loligo vulgaris	6																			
Sardina pilchardus	5	1	1	1			20.0[b]	20.0[b]	20.0[b]			1			1			0.2		
Scorpaena porcus	5	2					40.0[b]													
Todarodes sagittatus	5	3	1		1	3	60.0	20.0		20.0	60.0		1	8		1	2.7	0.2	0.2	1.6
Illex coindetii	4	2	1	1			50.0	25.0	25.0			1			1			0.25		
Spicara spp.	3	2				2	66.6[b]							25						
Serranus cabrilla	3	3				3	100[b]							111						
Pagellus acarne	2	2				1	100[b]							42						
Sardinella aurita	2																			
Hoplosthetus mediterraneus	2																			
Gobius cobitis	2	2				1	100[b]							1						
Ophisurus serpens	2	1					50.0[b]													
Uranoscopus scaber	1	1				1	100[b]							5						
Cyprinus carpio	1	1				1	100[b]							1						
Trigloporus lastoviza	1	1				1	100[b]							12						
Phycis blennoides	1																			
Eledone cirrhosa	1																			
Total	369	230	90	88	5	174	62.3	24.4	23.8	1.35	47.2	712	9	3,002	8.09	1.80	17.2	1.93	0.02	8.13

[a] AN, Anisakidae; An. spp., *Anisakis* spp.; An I, *Anisakis* spp. type I; An II, *Anisakis* spp. type II; Hyst, *Hysterothylacium* spp. The following formulas were used to calculate prevalence (P), mean intensity (MI), and mean abundance (MA): P = number of infected specimens/total number of specimens examined × 100; MI = number of larvae/number of infected specimens; MA = number of larvae/total number of specimens examined.

[b] Data not considered due to insufficient sampling.

TABLE 2. *Prevalence and mean intensity of* Anisakis *larvae in muscles of sampled teleosts*

Fish species	No. of specimens	No. of infected specimens	P (%)	No. of larvae	MI
Trachurus spp.	59	13	22.0	26	0.84
E. encrasicolus	52	16	30.8	34	1.9
M. merluccius	50	5	10.0	6	0.4
M. poutassou	16	10	62.5	20	1.4
S. scombrus	9	3	33.3	9	1.8
Other spp.	183				
Total	369	47	12.7	95	1.06

[a] The following formulas were used to calculate prevalence (P) and mean intensity (MI): P = number of infected specimens/total number of specimens examined × 100; MI = number of larvae/number of infected specimens.

particular in *M. poutassou, S. scombrus, Engraulis encrasicolus,* and *Trachurus* spp., but not in any cephalopods.

Bibliographic data on the P, MI, and MA values for anisakid nematodes in the Mediterranean were compared with our results, which were similar to those of Pasolini et al. *(42)* for the prevalence of Anisakidae larvae in *Trachurus* spp., *S. scombrus,* and *M. poutassou* caught in the Mediterranean. However, they differed for *Merluccius merluccius, Z. faber, E. encrasicolus,* and *Mullus* spp., which were found to be less infected by anisakids, with *E. encrasicolus* having a P about five times lower than Pasolini's values. Valero et al. *(53)* in their study in Motril Bay found a P of Anisakidae in *M. poutassou* about nine times lower. By contrast, Costa et al. *(13)* and Costa *(12)* in their studies of fish caught off the Sicilian coasts and sold in local markets found lower P values of *Anisakis* in both *M. merluccius* and *S. scombrus* than we did in the present work. However, Costa et al. *(13)* found higher values of MI and MA in *M. merluccius,* which indicates a greater number of larvae per fish. They also found no *Anisakis* in *M. barbatus, M. surmuletus, Z. faber,* and *E. encrasicolus*. Only in *T. trachurus* did they report a slightly higher P of *Anisakis (12).* Fioravanti et al. *(23)* in their study in the Adriatic Sea found lower P for *Anisakis* in *S. scombrus* and in *T. trachurus* than the P found in the investigation presented here. Anastasio et al. *(3)* carried out research into the P of *Anisakis* in *E. encrasicolus* taken from the Gulf of Naples, and their results showed a P about 70 times lower than ours. MacKenzie et al. *(34)* studied a large area (southern Sicilian coastal waters, the Adriatic Sea, and the Tyrrhenian Sea) for the P of *Anisakis*. They found a total P in *T. trachurus* that was almost double that recorded in the present study, whereas the same fish species had 50 times lower values in southeast France and Balearic islands *(34).* Studies on type I and type II *Anisakis* larvae and relative epidemiological data were performed by Rello et al. *(46)* in different Mediterranean localities, by Gutiérrez-Galindo et al. *(27)* in Tarragona (Spain) and by Valero et al. *(52, 53)* off the Andalusian coasts. In comparison to these studies with regard to *Anisakis* type I, the present study reports mostly higher P, MI, and MA. By contrast, with regard to *Anisakis* type II, Valero et al. *(52)* recorded a P of 1.6% in *M. merluccius*. In the research presented here, this fish was not infected by this type of larva. In *M. poutassou,* Valero et al. *(53)* found *Anisakis physeteris,* the most diffused type II larva in the western Mediterranean, to have a P of 2.66%, while in this study higher P levels were found. Regarding *Hysterothylacium* spp., P values found in the present work were higher than the above-mentioned ones. In addition, some articles did not detect *Hysterothylacium* larvae *(13, 27, 30).* Lecis et al. *(30)* and Rello et al. *(46)* found P values for *Hysterothylacium* larvae in *M. barbatus, M. surmuletus,* and *E. encrasicolus* higher than those in the present research. Only Fioravanti et al. *(23)* reported data regarding coinfection (*Anisakis* and *Hysterothylacium* larvae) in *T. trachurus* and prevalences that were much lower than those obtained in our research. The same authors reported a P of 1.5% in *S. scombrus,* but in the present study no coinfection was observed for this teleost species.

Few studies have been carried out exclusively in Sardinia. Lecis et al. *(30)* analyzed the presence of anisakids on fish caught only in the Gulf of Cagliari, while in the present work specimens were caught in many different Sardinian coastal areas and lagoons. They found higher P values for *Anisakis* type I and II larvae in *M. merluccius* and in *T. trachurus*. Lecis et al. *(30)* found *Hysterothylacium* larvae with a higher P than the present study only in *M. barbatus* and *M. surmuletus*. No *Hysterothylacium* larvae were found in other fish species. This is probably due to the parasite detection method that they used, which was not explained in detail in their study. *Hysterothylacium* larvae are, indeed, generally smaller than *Anisakis* and thus cannot always be easily detected by visual inspection.

In cephalopods, high prevalences of Anisakidae were recorded only in *T. sagittatus* (60%) and in *Illex coindettii* (50%), although only few specimens of both species were analyzed (Table 1). In the remaining species, P was very low, which suggests that infection levels are low in this class of organisms. This conclusion is reinforced by the low P of *Anisakis* in the two above-mentioned species (20 and 25%, respectively). Moreover, no *Anisakis* larvae were found at the muscular level (Table 2).

Only a few authors have studied the presence of anisakids in cephalopods in the Mediterranean. Among these, Pasolini et al. *(42)* reported a P of 22% in *T. sagittatus,* which is lower than our results.

Anisakis was found in the muscles of only five species of teleosts, with the highest incidence (62.5%) in *M. poutassou* (Table 2). These data indicate that this species is particularly dangerous for nematode transmission. It has also been shown that larvae migrate after death of the host into the flesh of ''fatty'' species (e.g., herring and mackerel) but not into that of ''nonfatty'' ones (e.g., blue whiting and walleye pollock) *(48).* This suggests that *Anisakis* larvae probably also migrate at the muscular level in *M. poutassou* while the host is alive.

In other similar studies, Fioravanti et al. *(23),* Gutiérrez-Galindo et al. *(27),* Costa et al. *(13),* and Rello et al. *(46)* found that larvae in the muscular tissues of teleosts had a lower P than those found in our work. This discrepancy may

be due to the different detection methods used. The visual examination and observation method, artificial digestion method, and candling method used in these studies may have led to lower prevalences. For example, Huang *(29)* states that candling is effective when fillets are fresh and thin, whereas when they are over 0.5 cm thick, *Anisakis* larvae become difficult to find. In addition, Levsen et al. *(31)* found that only 7 to 10% of the *A. simplex* larvae were detected by candling in the fillets of different fish species.

As already stated, the samples arrived refrigerated at the laboratory within 12 h after fishing and were not eviscerated. As a result, *Anisakis* larvae could have had time to migrate to the muscles. Indeed it is still not clear under what conditions and in what species postmortem migration occurs *(21)*. The conditions in which specimens were shipped to the laboratory were similar to those of the products sent to fish markets, which allowed us to obtain data under realistic conditions.

Nevertheless, it must be said that larvae were found only in the muscles of five teleost species and not in cephalopods. Thus, given that the edible part of these organisms is the muscle, cephalopods are safer than fish. As a result, the implications to public health may be considered low.

Furthermore, the definitive and preferential hosts of *Anisakis pegreffii* (i.e., *Delphinus delphis, Tursiops truncatus,* and *Stenella coeruleoalba*) are regularly recorded in Sardinian waters and in particular in the ''Sanctuary for Cetaceans,'' which has the largest variety of species of cetaceans in the western Mediterranean *(26, 38, 39, 41)*. This would confirm the ''anisakid paradox'' cited by Mattiucci *(37),* which states that a healthy marine ecosystem has high anisakid nematode infection levels.

Until some years ago, *Anisakis* worms were considered typical of blue fish (e.g., Atlantic mackerel, European anchovies, and Horse mackerel), but recently they have also been found in almost all fish and mollusk species *(9)*. This is probably not because of the greater diffusion of the parasite but rather because more effective detection methods have been developed.

Some EU Regulations (EC No 853/2004, EC No 854/2004, and EC No 2074/2005) oblige food business operators to carry out checks, such as ''visual inspection'' and ''candling'' on fishery products *(17–19)*. In this work it has been observed that visual inspection may not be an adequate way to detect nematode larvae, especially in muscles. Here it should be said that the visual inspection commonly used in fish markets may not guarantee that the fish products are completely safe. This is because they consist of random sampling and depend on the operator's experience and the brightness of the inspection environment *(21)*. These same factors could also lead to confusion between products infected only by *Hysterothylacium,* a nonzoonotic nematode, and products infected by zoonotic nematodes.

In conclusion, an important prevalence of Anisakidae, and particularly *Anisakis,* was observed, with higher values than those recorded in other areas of the Mediterranean. Epidemiological data on *Anisakis* diffusion are considered more and more important in legal terms, since Regulations EC No 853/2004 and EC No 2074/2005 state that food business operators are not obliged to apply freezing treatments on fishery products if the epidemiological data show that there are no parasites in the fishing grounds *(17, 20)*. However, the data obtained in this study on the diffusion of this nematode are not very encouraging. Fish caught in Sardinian waters seem to be potentially hazardous in terms of spreading *Anisakis* larvae. Thus, measures should be taken to minimize human risk such as, for example, establishing adequate information campaigns addressed to both sanitary service personnel and consumers. It may also be useful to evaluate whether the presence and/or quantity of these parasites in these areas changes over time, as knowledge about trends on nematode distribution over time is lacking and there are no long-term sampling plans in Europe at present *(21)*.

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