Reevaluation of Atlantic anchovies (*Engraulis encrasicolus*) as a main risk factor for sensitization to zoonotic nematodes in Spain

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Abstract

Previous clinical studies pointed out the consumption of raw *Engraulis encrasicolus* in vinegar (“boquerones”) as the main potential risk source for anisakiosis and IgE sensitization to *Anisakis* in Spain. In a recent work that developed a quantitative risk assessment (QRA) model for the anchovy value chain in Spain, the number of predicted anisakiosis cases requiring medical attention was reported to be about 8000 per year. Conversely, most epidemiological surveys for this fish species taken from the Spanish markets showed parasite-free fish lots or parasite prevalence around 10%. We analyzed 956 anchovies collected on board from Spanish NE Atlantic grounds to re-evaluate the importance of this species as a potential source of risk exposure to zoonotic and allergy diseases.

Epidemiological results revealed an overall mean prevalence for zoonotic nematodes (genera *Anisakis* and *Hysterothylacium*) of 64.33% (14.75% in the flesh), ranging up to 87% (28.65% in the flesh) in one of the lots, representing the highest values ever reported for this species in any EU fish production value chain. This finding is of public health concern in Spain considering its traditional raw consumption as homemade “boquerones” in vinegar. An enhanced risk communication focus on control measures may clearly contribute to avoid this biohazard in seafood.

1. Introduction

Nematodes are one of the parasite group primarily considered as responsible for fishery product-borne parasitic diseases. These are either caused by an infection following ingestion of viable parasites, or as an allergic (hypersensitivity) reaction against parasite antigens, as in the case of the genus *Anisakis* (EFSA, 2010). Fishborne larval nematodes belonging to the family Anisakidae are widespread in fish populations worldwide (Nadler et al., 2005; Mattiucci & Nascetti, 2008; Karpiej et al., 2013). Species from the three anisakid genera *Anisakis*, *Pseudoterranova* and *Contracaecum* have heteroxenous complex life cycles involving marine mammals as final hosts, invertebrates as intermediate hosts and fish as transport or paratenic hosts (Koie and Fagerholm, 1995; Koie et al., 1995, EFSA, 2010). Humans may become accidental hosts for anisakids if they consume raw or undercooked parasitized fish. Infection is known as anisakidosis (anisakiosis, pseudoterranovosis and contracaecosis if we wish to be genus specific) and is often associated with acute gastrointestinal symptoms such as abdominal pain, diarrhea, nausea and vomiting, although patients range from being asymptomatic to requiring emergency room care. In addition, allergic reactions are often reported, even though life-threatening allergic reactions (Audicana & Kennedy, 2008; EFSA, 2010; Nieuwenhuizen & Lopata, 2013, Buchmann & Mehrdana, 2016).

Human infections usually involve the genera *Anisakis* and *Pseudoterranova* (Yu et al.; 2001; Audicana et al., 2002; Sukuki et al, 2010; Arizono et al., 2011, 2012; Cavallero et al., 2016), but also *Contracaecum* was recognized as responsible for human gastrointestinal infections in a few cases (Buchmann & Mehrdana, 2016). Numerous investigations of clinical cases have documented the pathogenicity of the anisakid worm larvae in man which has called for further investigations using experimental animals in order to further characterize pathogenesis. Rats, rabbits, dogs and pigs may be applied for experimental infections and studies have shown that several species of *Anisakis*, *Pseudoterranova decipiens*, *Contracaecum osculatum* and *Contracaecum multipapillatum* penetrate the mucosa of the gastro-intestinal tract (Desowitz, 1986; Vidal-Martínez et al., 1994; Barros et al., 2004; Romero et al., 2014; Strom et al., 2015; Buchmann & Mehrdana, 2016).

Allergic reactions to anisakid worms have been only related to the genus *Anisakis* (EFSA, 2010). A range of allergens have been isolated from *A. simplex* and these can cause disease in sensitized patients even without exposure to the live worm (Caballero et al., 2011; Arcos et al., 2014; Faeste et al., 2014). Thus, ingestion of processed fish products with heat and frost stable worm allergens have been reported to elicit allergic reactions in consumers (Audicana et al., 1997) but it is generally believed that an active infection with a live penetrating *Anisakis* larvae

is needed to sensitize and establish the allergic condition in patients (Alonso-Gómez et al., 2004). Allergens released from *Pseudoterranova* or *Contracaecum* have not been described in detail but due to biological similarities to *Anisakis*, it cannot be ruled out that the two species release compounds with an allergenic potential (Buchmann & Mehrdana, 2016).

Another very common nematode in marine fish is the Raphidascaridae genus *Hysterothylacium*. The adult worms mostly parasitize the digestive tract of fishes whereas the larval stages live in different tissues of numerous fish species and in over 100 invertebrate species from seven phyla, which act as intermediate and/or paratenic hosts (Deardoff & Overstreet, 1980; Koie, 1993; Marcogliese, 1996; Navone et al., 1998). *Hysterothylacium* has been rarely reported as associated to gastrointestinal symptoms or allergies in humans (Yagi et al., 1996), but recent data showed a case of invasive gastroallergic anisakidosis caused by the third stage larvae of *H. aduncum* (González-Amores et al., 2015). Also, Fernández-Caldas et al. (1998) and Valero et al. (2003) demonstrated that this parasite has antigens in common with *A. simplex*, which may have important health implications regarding allergic processes resulting from ingestion of fish parasitized by these nematodes (Rello et al., 2008).

Noticeable cases of anisakiosis and allergic reactions to *Anisakis* reported in Spain have increased dramatically in the last decade. One of the main risk factor described is the ingestion of homemade raw anchovies as “boquerones en vinagre” (i.e., raw anchovies in vinegar, *Engraulis encrasicolus*), (Rubio et al., 2000; López-Serrano et al., 2000; Valiñas et al., 2001; Repiso-Ortega et al., 2003; Alonso-Gómez et al., 2004; González-Quijada et al., 2005; Del Rey Moreno et al., 2008; Puente et al., 2008), a very popular dish in Spain and other Mediterranean countries. In a very recent work by Bao et al. (2017), a QRA model was developed for the anchovy value chain, and the number of annual anisakiasis cases requiring medical attention was predicted between 7700 and 8320, suggesting that previously reported figures of 500 anisakiosis per year in Europe (Arizono et al., 2012) or 20 cases on average per Europe country per year (D’amico et al., 2014) are a considerable underestimate.

The marked role of anchovies sold in Spanish markets in anisakiosis seemed hard to understand, as most epidemiological surveys showed parasite-free fish lots or parasite prevalence around 10% (Cuellar et al., 1991; Pereira Bueno, 1992; López Gimenez & Castell Monsalve, 1994; Viu et al., 1996; Osanz Mur, 2001; Valiñas et al., 2001; Valero et al., 2004).

Anchovy is a small pelagic species, with a short life cycle, seasonally caught in NW Spanish waters by purse-seine fleet. This species is greatly appreciated in Spain, being one of the most economically important in national fish markets. In fact, 73% of the Spanish households stated

that regularly buy and consume this product (<http://www.mercasa.es>). Although European Union and Spanish Regulations require food establishments to freeze fish destined for raw consumption, “boquerones en vinagre” still carries the risk of anisakiasis for home consumption (Rello et al., 2009; Bao et al., 2017). We herein re-evaluate the exposure level to this risk.

2. Materials and methods

2.1. Sampling and parasite detection

Experimental design was carried out according with the general rules for parasite surveillance established in the PARASITE Project (EU-GA312068) (Figure 1). A total of 956 anchovies in three lots (mean overall weight of 21.97 g.) from ICES VIIIc were examined for anisakids. Fish were placed in ice after catching until landing and transport to the lab, where it was immediately frozen to prevent nematode migration. After thawing, individual fish were gutted and dissected in left and right fillets and then each fillet was divided into four pieces: anterodorsal (AD), posterodorsal (PD), anteroventral (belly flaps, BF) and posteroventral (PV). Each of the eight muscle pieces obtained from every fish were placed in transparent plastic bags and then compressed to 2 mm thickness by using a hydraulic press Mega 3D Ton KMG-30 (Melchor Gabilondo, S.A., Spain). Pressed fillets were visually inspected under a UV light source in dark conditions, using a Vilbert Lourmat CN-15LC Cabinet (Vilbert Lourmat, Marne La Vallée, France) at 300-400 nm wavelength measure range, 365 nm peak excitation and 100 $\mu\text{W}/\text{cm}^2$ peak irradiance. Viscera and muscle pieces with visually recognized massive infections were digested enzymatically, to ensure detection of all nematode present, following Llarena-Reino et al. (2013). Nematode samples were stored frozen ($-80\text{ }^{\circ}\text{C}$). All the data for every individual fish and for every nematode sample was collected and coded in a BioBank platform specifically designed within the PARASITE project, which ensures sample and data traceability.

Quantitative descriptors as Prevalence (P), Mean Intensity (I) and Mean Abundance (A) were calculated according to Bush et al. (1997). Additionally, those descriptors were also obtained for the viscera (Pv, Iv and Av) and especially for musculature (Pm, Im, Am), as the edible part of the fish is reliable as a source of hazard exposure for human infection with these nematodes.

2.2. Molecular identification

A subsample of 61 anisakid larvae from anchovies was identified using molecular methods (42 previously classified as *Hysterothylacium* sp. and 19 of *Anisakis* sp.). Genomic DNA purification was performed employing NucleoSpin Tissue Kit (Macherey-Nagel, Easton, PA), according to the manufacturer's protocol for isolating genomic DNA from human or animal tissue and cultured cells. DNA quality and quantity was checked in a spectrophotometer Nanodrop® ND-2000 (Thermo Scientific). The entire ITS region (ITS-1, 5.8S rDNA gene and ITS-2) was amplified using the forward primer NC5 and reverse primer NC2 (Zhu et al., 2000). PCR reactions were performed in a total volume of 25 µl containing 1 µl of genomic DNA (100 ng), PCR buffer at 1x concentration, 0.3 µM primers, 0.2 mM nucleotides and 0.025 U. µl⁻¹ KAPA Taq DNA polymerase (KAPABIOSYSTEMS). The PCR assays were carried out in a Tgradient thermocycler (Biometra), under the following reaction conditions: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min 15 s at 72 °C, followed by 7 min at 72 °C. A negative control (no DNA) was included in all PCR amplifications. The PCR products were separated on a 2% agarose gel in Tris acetate EDTA buffer, stained with Red Safe and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories). PCR products were cleaned for sequencing using ExoProStar™ 1 Step (GE Healthcare, NJ, USA) for 15 min at 37 °C, followed by inactivation for 15 min at 80 °C. Sequencing was performed in a specialized service (Secugen, Madrid) and the chromatograms were analyzed using ChromasPro v.1.41 Technelysium Pty Ltd. All generated sequences were searched for identity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA).

Phylogenetic analysis was performed using the sequences obtained in this study and others available from GenBank. Multiple alignments based on ITS region of nematode parasites were performed using Clustal W (Thompson et al., 1994) included in MEGA6 software. Minimum evolution tree was constructed and reliability of the inferred tree was tested by 1000 bootstrap replicates. The analysis of the ITS1-5.8S-ITS2 region included ten sequences obtained in this study and the following sequences deposited in GenBank: *A. simplex* (KF512906, KC663498, KF953969), *A. pegreffii* (KJ011495, KF923927, JN968597), *A. physeteris* (JQ912693, JN005754), *Hysterothylacium* sp. (JQ798963), *H. deardorffoverstreetorum* (JF730201), *H. fabri* (KU948637, KC852206) and *H. aduncum* (JX845135, KP979761, KU306720). The sequence of *Ascaridia columbae* (KF147909) was used as outgroup.

3. Results

3.1. Demographic data

In the sampled anchovies members of the genus *Anisakis* and *Hysterothylacium* were recognized as the two different morphological types of larval nematodes present both in visceral and muscular locations. Nematode total prevalence was 64.33% (ranging between lots from 29.58% to 87%), with intensity reaching 3.84 and abundance of 2.47. For muscular parasitisation, prevalence was of 14.75% (7.04-28.65%), intensity 1.28 and abundance 0.19. The 2.40% of the anchovies examined presented only muscular parasitisation, reaching the 4.22% in one of the lots (Table 1). *Anisakis* spp. appeared in the 57.32% of the anchovies (Pm= 13.70, Pv= 54.60%) and *Hysterothylacium* sp. was identified in the 16.63% of the samples (Pm= 1.25% and PV= 15.79%). Co-infection was recorded in 14.96% of parasitized anchovies.

3.2. Molecular identification

All sequences obtained in this study (n=61) shared 100% nucleotide identity with nematode species deposited in the GenBank belonging to *Anisakis simplex* (n= 17 sequences), *Anisakis pegreffii* (n= 2) and *Hysterothylacium aduncum* (n= 42). Phylogenetic analysis was performed with a total of 699 sites and 26 sequences. ITS tree showed that *Anisakis* species were grouped in the same clade with high bootstrap value (99%) (Fig.2). Within this clade, the sequences obtained were grouped into subclades of *A. simplex* and *A. pegreffii*. The sequences identified as belonging to *H. aduncum* were grouped in the same clade as other known *H. aduncum* sequences with bootstrap values of 99%.

4. Discussion

4.1. Demographic data

Some disagreement was found when we compared the epidemiological results obtained for anchovies in our study with those previously recorded in the literature (Table 1). Remarkably, *Anisakis* prevalence reached up to 87% in one of the fish lots, only comparable with those recently obtained by Buselic et al. (2015), Mladineo et al. (2012) and Mladineo & Poljack (2013) in the Croatian Eastern Adriatic Sea, and for Cipriani et al. (2016) in the Central Adriatic Sea (Italy). Muscular infection data are there far lower than in our samples, except for the recent data by Cipriani et al. (2016) that also found high levels of muscular parasitisation from

the Adriatic Sea, an area that seems a “hot spot” at the Mediterranean, as it has been described for the Grand Sole in North Atlantic waters (Llarena-Reino et al., 2013). Data of muscular prevalence for anisakids in one of our lots was 28.65% (ranging from 28.65% to 7.04%, with a total Pm= 14.75%), which represents the highest infection values ever described. Of noteworthy is the comparison between our results and those from Valiñas et al. (2001) who reported parasite-free fish in 650 anchovies obtained from the same fishing ground.

Reasons for the above heterogeneity of epidemiological results have many sources, especially those related to different geographical areas/fishing grounds and seasons in the same fish host species (Mattiucci et al., 2002, 2004; Tejada et al., 2006; Serracca et al., 2014). It has been noted in several fish species that catching locality had by far the strongest effect on larval infection probability (Levsen and Karl, 2014; Rello et al., 2009), and thus reasons for this dissimilarity may be attributable to the different geographical fish origin but also to the complex interactions of different host-related and environmental conditions, even to inter-annual variability. It is also likely that such differences reflect discrepancies between detection methods. In the case of *Hysterothylacium*, detection by visual inspection of larvae has been difficult, as their size at larval stage is much smaller than *Anisakis*, and the smaller ones did show also little fluorescence under UV light. Thus, it should not be discount that demographic infection values can be underestimated for this genus.

4.2. Molecular identification

Three nematode species were identified (*A. simplex*, *A. pegreffii* and *H. aduncum*). The proportion of *A. simplex* vs. *A. pegreffii* was 17:2 in the 19 nematodes analyzed. This is sounds as *A. simplex* is the most common species found in all samples from NE Atlantic waters, with its relative proportion decreasing from north to south. In contrast, *A. pegreffii* was the most prevalent species in the Mediterranean Sea. Mixed infections of *A. simplex* and *A. pegreffii* were described from south of Ireland to the Mediterranean (Mattiucci et al., 2008), including so all the Atlantic Galician coast.

It is also worth noting the detection of *Hysterothylacium* larvae in flesh from anchovies. Cipriani et al. (2016) never found *H. aduncum* larvae in the musculature of 1300 anchovies, even at those temperatures and incubation times that increased the burdens of *A. pegreffii* in fish flesh and decreased the number of larvae in viscera. Cavallero et al. (2015) found larvae of *Hysterothylacium* and *Anisakis* in anchovies (N=4350), but none on musculature. This muscular

presence of *Hysterothylacium* has been only described, to our knowledge, by Rello et al. (2008) in sardines, *Sardina pilchardus*.

4.3. Conclusions

Among anisakids it has been described that *A. simplex*, *A. pegreffii* and *P. decipiens* are the species mainly responsible for anisakidosis and gastroallergic reactions in humans (Arizono et al., 2011; 2012; Mattiucci et al., 2013; Cavallero et al., 2015). Rarely, anisakidosis symptoms have been found in association with larvae of the Anisakidae genus *Contracaecum* and the Raphidascaridae genus *Hysterothylacium* in gastric/intestinal tract (Shamsi and Butcher, 2011; Yagi et al., 1996). The latter genus has been commonly considered not pathogenic to humans, but recent data showed a case of invasive gastroallergic infection caused by the third stage larvae of *H. aduncum* (González-Amores et al., 2015). That finding, together with the presence of this parasite in the musculature of anchovies leads to the necessity to reevaluate the exposure level to risk associated to ascaridoid nematodes recorded in anchovies. As we have pointed out before, different epizootiological investigations revealed low *Anisakis* prevalence in anchovies, but some of these studies showed also quite high *Hysterothylacium* prevalence (Fiovaranti et al., 2003, 2006; Rello et al., 2009; Fiovaranti & Gustinelli, 2009; Angelucci et al., 2011; Cavallero et al., 2015). It could not be therefore discarded a role of *Hysterothylacium* in the “anisakiosis” reported cases associated to anchovies consumption.

Since anchovies are alleged to be responsible for most cases of anisakiosis in Spain (Lopez-Serrano et al., 2000; Valiñas et al., 2001; Repiso-Ortega et al., 2003; González- Quijada et al., 2005; Del Rey Moreno et al., 2008; Puente et al., 2008), the high prevalence of zoonotic nematodes in these species is of great public health concern, considering that flesh prevalence may reach 29% in pre-harvest condition, besides the traditional raw consumption of these fish in Spain. Although postmortem larval migration from viscera to flesh has been demonstrated in anchovies (Cipriani et al., 2016), this process can be discarded in our samples. Migration is highly influenced by the freshness of the sample, being accelerated with the time lapsed from the moment of fish catch (Mladineo et al., 2001; Suzuki et al., 2010; Serracca et al., 2014; Cipriani et al., 2016), and with temperatures above 2 °C (Cipriani et al., 2016). In our study anchovies were brought to the laboratory within a few hours after being caught, always on ice, and immediately frozen, precluding the possibility of postmortem larval migration.

Overall, a reevaluation of the risk posed by the consumption of anchovies is needed. An enhanced risk communication focus on control measures may clearly contribute to avoid or at least minimize this biohazard in seafood.

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521

522 **Table 1:** Demographic infection values obtained in our work for *Engraulis encrasicolus* with comparison
523 with previous published data from other European Areas. P= Prevalence, I= Mean Intensity; A= Mean
524 Abundance, Pm= Muscular Prevalence, Im= Muscular Mean Intensity; Am= Muscular Mean Abundance.
525 These parasitation indexes in our work were calculated according Bush et al. (1997). N= number of
526 individuals analyzed

527

Area	Prevalence of <i>Anisakis</i>	Reference	Observations
Paris (France) Market	3%	Huang (1988)	
North East Cantabric Sea	25% in males 35% in females	Marigómez et al. (2006)	N= 136 males and 125 females
South East Cantabric Sea	36% in males 50% in females	Marigómez et al. (2006)	N= 157 males and 105 females
Galician Coast (Ría de Arosa)	0%	Valiñas et al., 2001	N=650
Galician Coast ICES VIIIc 43°28'N 8°25'W 22/08/2014	P= 87.08% <i>Anisakis</i> Pm= 28.65% Pv= 83,15% 3.93% showed only flesh parasitisation	Present study	N= 178 (medium weight: 26.30 g) UV Press Method I= 5.4 Im= 1.35 Iv= 5.19 A= 4.7 Am= 0.39 Av= 4.31
Galician Coast ICES VIIIc 22/04/2015	P: 62% Anisakids 52.54% <i>Anisakis</i> sp. 22.17% <i>H. aduncum</i> Pm: 12% anisakids 10.59% <i>Anisakis</i> 1.69 % <i>H. aduncum</i> Pv: 60.17% Anisakids 50.28% <i>Anisakis</i> sp. 21.04% <i>H. aduncum</i> 1.84% showed only flesh parasitisation by anisakids Coinfestation in 20.50% of the anisakid positive	Present study	N= 708 (medium weight: 20.49 g) UV-Press Method Anisakids I= 3.35 Im= 1.23 Iv= 3.21 A= 2.08 Am= 0.15 Av= 1.93 <i>Anisakis</i> : I= 3.17 Im= 1.23 Iv= 3.05 A= 1.66 Am= 0.13 Av= 1.53 <i>H. aduncum</i> : I= 1.86 Im= 1.08 Iv= 1.87 A= 0.41 Am= 0.02 Av= 0.39
Galician Coast ICES VIIIc 43° 22'N 08° 35' W 09/09/2015	P: 30% anisakids 30% <i>Anisakis</i> 2.86% <i>H. aduncum</i> Pm: 7.14% <i>Anisakis</i> 0% <i>H. aduncum</i> Pv: 25.35% anisakids 2.86% <i>H. aduncum</i> 4.22% showed only flesh parasitisation by anisakids Coinfestation in 9.52% of the anisakid positive	Present study	N= 70 (medium weight: 27.38 g) UV Press Method Anisakids: I= 2.48 Im= 1.4 Iv= 2.5 A= 0.74 Am= 0.10 Av= 0.64 <i>Anisakis</i> spp.: I= 2.33 Im= 1.4 Iv= 2.33 A= 0.7 Am= 0.1 Av= 0.6 <i>Hysterothylacium aduncum</i> : I= 1.5 Im= 0 Iv= 1.5 A= 0.04 Am= 0 Av= 0.04
Merca Bilbao (Basque Country Market)	2,08%	Pereira Bueno (1992)	N= 48
Castilla-La Mancha (Spain) Markets	0%	López Gimenez & Castell Monsalve (1994)	
Zaragoza (Spain) Markets	0%	Viu et al. (1996)	N= 110

			Visual inspection and peptic digestion
Spain (different localizations)	3-34%	Coronilla Pérez et al. (1999)	
Golfo de Cádiz and Strait of Gibraltar (Eastern Atlantic South Spain)	13,13% <i>H. aduncum</i> 4.30%	Rello et al. (2009)	N= 396 Pepsin digestion of viscera and flesh separately
Mediterranean Sea (Spain)	0%	Cuellar et al. (1991)	
Mediterranean Sea (Andalucía, Spain)	4.17% Anisakids 1.39% <i>Anisakis</i> 2.28% <i>H. aduncum</i>	Valero et al. (2004)	N= 72 Pepsin digestion
Mar de Alborán (Mediterranean Sea, Spain)	1,39% <i>Anisakis</i> 2.78% <i>H. aduncum</i>	Rello et al. (2009)	N= 72 Pepsin digestion of viscera and flesh separately
Cataluña (Mediterranean Sea, NE Spain)	0%	Osanz Mur (2001)	N= 153 Visual inspection and peptic digestion for fish flesh
	1.91% 37.58% <i>H. aduncum</i>	Rello et al. (2009)	N= 157 Pepsin digestion of viscera and flesh separately
Tarragona (Mediterranean Sea, NE Spain)	0%	Gutierrez Galindo et al. (2010)	N= 153 Visual inspection and pepsin digestion
MercaGranada (Granada Market, SE Spain)	9.34% <i>Hysterothylacium</i> 24.45%	Rello, 2003	
Córdoba Market (extracted usually from Golfo de Cádiz and Mediterranean Sea, exceptionally from Atlantic Ocean)	5,6%	De la Torre Molina et al. (2000)	N=269 Visual inspection
Málaga (Spain) Market	0%	Fernández Meléndez (1999)	
Golfo de León (Mediterranean Sea France)	3,88% 68.93% <i>H. aduncum</i>	Rello et al. (2009)	N= 103 Pepsin digestion of viscera and flesh separately
Mediterranean Sea (Italy) Golfo di Napoli	0,51%	Anastasio et al. (2007)	
Mediterranean Sea, Ligurian Coast (Italy)	0%	Poirè et al. (1988)	
	21,88% 70.31% <i>H. aduncum</i>	Rello et al. (2009)	N= 64 Pepsin digestion of viscera and flesh separately
	0.8% <i>Anisakis pegreffii</i>	Serracca et al. (2014)	N= 1050 Inspection by candling
Mediterranean Sea (Italy) Northern Sardinia	77.0% Anisakids 34.6% <i>Anisakis</i> 67.3% <i>Hysterothylacium</i> spp.	Angelucci et al. (2011)	N= 52 Visual inspection and peptic digestion Mean Intensity <i>Anisakis</i> : 3.6 Mean Abundance <i>Anisakis</i> : 1.25
Tyrrhenian Sea (Central Italy)	0%	Sandulli et al. (1988)	
	0%	Renon & Malandra (1991)	
Tyrrhenian sea (Central Italy)	2.3% Anisakidae <i>A. pegreffii</i> (1%), <i>H. aduncum</i> (0.7%) and hybrid <i>A. pegreffii/A.simplex</i>	De Liberato et al. (2013)	Microscopic examination and peptic digestion
Tyrrhenian sea (Tuscany)	37.7%	Cavallero et al. (2012)	N= 61 Search only in the abdominal cavity
South Tyrrhenian Sea	7.1%	Marrone et al. (2007)	
Sicily (Italy) Market	0% 16.7% <i>Hysterothylacium</i>	Costa et al. (2008a,b)	N= 6 Visual inspection and with translumination
Sicily Coast	2.69% <i>A. pegreffii</i>	Costa et al. (2010)	N= 310
North Adriatic Sea, Venetian Coast (Italy)	0.5% <i>Anisakis</i> 27.60% <i>H. aduncum</i>	Cavallero et al., 2015	N= 4350 <i>Anisakis</i> : I= 1.25 A= 0.006 <i>H. aduncum</i> I= 1.04 A= 0.287 Peptic digestion of a flesh fragment Viscera examined using a 5-diopter (2.25x) bright lens and by Baermann test
Adriatic Sea (Italy)	0%	Renon & Malandra (1991)	
Adriatic Sea (Italy) (fish market from Fano and Ancona). Fishing	53,1% anisakids 4,11% <i>Anisakis</i>	Fioravanti et al. (2003, 2006)	N=414

area from Cattolica to S. Benedetto del Tronto	0.9% only <i>Anisakis</i> 6.8% coinfection 92.3% <i>Hysterothylacium</i>		Visual inspection
Adriatic Sea (Italy). Cesenatico	0.2% 22.9% <i>Hysterothylacium</i>	Fioravanti & Gustinelli (2009)	
Adriatic Sea (Italy). Ancona	7.8% 56% <i>Hysterothylacium</i>	Fioravanti & Gustinelli (2009)	
Adriatic Sea (Italy). Giulianova	3.9% 24.9% <i>Hysterothylacium</i>	Fioravanti & Gustinelli (2009)	
Adriatic Sea (Italy). Vasto	3.5% 38.9% <i>Hysterothylacium</i>	Fioravanti & Gustinelli (2009)	
Adriatic Sea (Italy). Manfredonia	0% 9.8% <i>Hysterothylacium</i>	Fioravanti & Gustinelli (2009)	
Adriatic Sea (Italy). Fish market in San Benedetto del Tronto. Fishing area from Cattolica to S. Benedetto del Tronto	4,79% 1.23% <i>Hysterothylacium</i> 0.3% coinfection	Ciccarelli et al. (2011)	N=5696 Visual inspection Stational variation. Higher prevalence in May and October
Central Adriatic Sea (Italy)	<i>A. pegreffii</i> P= 87.0-96.0% Pm= 20.0-25.0% Pv= 87.0-95.0% <i>H. aduncum</i> (only in viscera) P= 27.0-32.0%	Cipriani et al. (2016)	N= 100 (x4 batches) <i>A. pegreffii</i> I= 6.63-8.09 Im= 1.20-1.40 Iv= 6.61-7.71 A= 6.46-7.61 Am= 0.27-0.30 Av= 6.18-7.34 <i>H. aduncum</i> A= 0.41-0.52
Adriatic Sea (Croatia)	76.1% Musculature 1.83%	Mladineo et al. (2012)	N= 4600 (different months in 2.5 years) Viscera examined under stereomicroscope. Fillets observed compressed between glasses with underneath light in a stereomicroscope.
Adriatic Sea (Croatia)	81.7%	Mladineo & Poljak (2013)	N= 120 Viscera examined under stereomicroscope. Musculature by UV-press method
Eastern Adriatic Sea	29.70% (3.20% in fillets)	Buselic et al, 2015	N=785 (2 years, 2013, 2014) UV Press Method <i>Anisakis pegreffii</i> and <i>Anisakis simplex</i> Mean Intensity: 2.41 Mean Abundance: 0.71 Mean Intensity in fillets: 1.12
Milan (Italy) Market	2,2%	Renon & Malandra (1993)	
Aegean Sea (Greece)	3.9% <i>A. pegreffii</i>	Chaligiannis et al. (2012)	N=77 Artificial peptic digestion Mean Intensity= 1.3 Mean Abundance= 0.05

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531 **Figure legends**

532 **Figure 1.** Experimental design for nematode detection carried out in PARASITE Project. AD:
533 anterodorsal, PD: posterodorsal, BF: belly flaps, anteroventral; PV: posteroventral

534 **Figure 2.** Minimum evolution tree of ITS1-5.8S-ITS2 sequence analysis showing the taxonomic position
535 of the nematode larvae that were found infecting *E. encrasicolus* (codes marked with *). Numbers at
536 branch nodes indicate bootstrap confidence values (%).

537

538

Figure 1

544 **Figure 2**
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