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Anisakis pegreffii (Nematoda: Anisakidae) in European anchovy *Engraulis encrasicolus* from the Mediterranean Sea: Fishing ground as a predictor of parasite distribution

Paolo Cipriani[a,b,*], Gian Luca Sbaraglia[b], Marialetizia Palomba[b], Lucilla Giulietti[a], Bruno Bellisario[b], Ivana Bušelić[c], Ivona Mladineo[c], Roberto Cheleschi[d], Giuseppe Nascetti[b], Simonetta Mattiucci[a,*]

[a] Department of Public Health and Infectious Diseases, Section of Parasitology, "Sapienza-University of Rome", P. le Aldo Moro, 5, 00185 Rome, Italy
[b] Department of Ecological and Biological Sciences (DEB), Tuscia University, Viterbo, Italy
[c] Laboratory for Aquaculture, Institute of Oceanography and Fisheries, Split, Croatia
[d] Fiorital S.p.A., Località Marittima, Venice, Italy

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ABSTRACT

European anchovy *Engraulis encrasicolus* represents one of the principal target species for commercial fishing in Europe. This fish is mostly consumed in different raw dish preparations, which represents a major risk for the fish-borne zoonosis anisakiasis. The present study provides a detailed epidemiological report on ascaridoid larvae in *E. encrasicolus* from several fishing areas in the Mediterranean basin. Between June 2013 and June 2016, a total of 4152 specimens of *E. encrasicolus* were obtained from 13 sampling areas. Parasitological analysis was carried out using the UV-press detection method. *Anisakis* larvae ($N = 547$), identified by diagnostic allozymes and analyses of partial sequences of the EF1 α-1 region of nDNA and mtDNA *cox2* gene, corresponded to *Anisakis pegreffii*. Additionally, sequence analyses of the ITS region of rDNA revealed the presence of *Hysterothylacium aduncum* larvae.

The levels of infection with *A. pegreffii* significantly varied between the selected fishing areas. Fish from the Central and South Adriatic Sea showed the highest levels of infection. In contrast, anchovies from Southern Sicily, Ionian and Alboran Seas, were uninfected. A great majority of *A. pegreffii* larvae (95.8%) were located in the body cavity, whereas only a small percentage of them (4.2%) were detected in the flesh of the fish. A significant positive correlation between fish length and abundance of *A. pegreffii* was observed. The fish body condition index and infection levels observed in different sampling areas did not correlate significantly. The infection levels by *H. aduncum* also showed a significantly uneven distribution between different fishing areas of the Mediterranean Sea, and no larval specimens of *H. aduncum* were detected in examined fish flesh. This study is the first Mediterranean-wide epidemiological assessment of infection in the viscera and flesh of *E. encrasicolus* by *A. pegreffii*, an important causative agent of human anisakiasis.

1. Introduction

European anchovy *Engraulis encrasicolus* (Linnaeus 1758) is a marine pelagic fish and one of the principal target species for commercial fishing in Europe.

Most of the Mediterranean anchovy catches are bound for local markets, where they are sold as fresh fish. These fish are small in size, and therefore, it is allowed to land, store and sell them ungutted and unfrozen. In Italy, Spain, Croatia and other European countries, *E. encrasicolus* represents the main fish species, which is traditionally consumed raw following simple home-made preparations (in lemon or vinegar, according to the regional/local traditional recipes, i.e., "marinated anchovies").

However, the consumption of raw anchovies, previously not exposed to freezing at −20 °C for 24 h, as is compulsory by the European regulation EC No 1276/2011, represents the main risk for contracting anisakiasis, a fish-borne zoonosis caused by

* Corresponding authors at: Department of Public Health and Infectious Diseases, Section of Parasitology, "Sapienza-University of Rome", P. le Aldo Moro, 5, 00185 Rome, Italy.
E-mail addresses: paolocipr@gmail.com (P. Cipriani), simonetta.mattiucci@uniroma1.it (S. Mattiucci).

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accidental ingestion of viable nematode larvae of the genus *Anisakis*. *Anisakis* species are parasites of marine organisms: crustaceans, such as euphausiids and mysids, are the first intermediate hosts (Gregori et al., 2015); fishes and squids are the second intermediate/paratenic hosts and cetaceans, mainly represented by dolphins and baleen whales, are the definitive hosts (Mattiucci and Nascetti, 2008). Larvae of *A. pegreffii* and *A. simplex* (s. s.) commonly infect the viscera and flesh of many teleosts fish species (Mattiucci and Nascetti, 2008). It should be noted that among the nine genetically characterized species of the genus *Anisakis*, only *A. pegreffii* and *A. simplex* (s. s.) have been reported as causative agents of human gastric (D'Amelio et al., 1999; Umehara et al., 2007; Fumarola et al., 2009; Mattiucci et al., 2013), intestinal (Moschella et al., 2004; Mattiucci et al., 2011; Mladineo et al., 2016) and gastro-allergic anisakiasis (GAA) (Mattiucci et al., 2013; Lim et al., 2015). In the fish host, larvae penetrate the intestinal wall, encoil on the surface of the internal organs and/or migrate into the flesh. This migration occurs both in live fish (i.e., *intra-vitam* migration) (Smith, 1984; Karl et al., 2002; Karl, 2008; Quiazon et al., 2011; Cipriani et al., 2016), and/or after the fish have been caught (i.e. *post-mortem* migration) (Smith and Wootten, 1975; Hauck, 1977; Šimat et al., 2015; Cipriani et al., 2016).

Data on the localization of *Anisakis* spp. larvae within the fish host are of crucial importance. The flesh represents the edible part of the fish, and therefore, larvae embedded in the flesh represent a potential source for human infection. Understanding the biology of larvae in the edible part of the fish will facilitate the assessment of risk for humans to contract this zoonosis. Several cases of human anisakiasis have been documented so far in Italy: in most of them, anamnestic information provided by the patients mentioned the consumption of raw marinated anchovies (Moschella et al., 2004; Mattiucci et al., 2011, 2013). The presence of third-stage larvae of *Anisakis* spp. in anchovies from the Mediterranean Sea and some Italian fishing grounds has been previously documented (Rello et al., 2009; Ciccarelli et al., 2011; Mladineo et al., 2012; De Liberato et al., 2013; Piras et al., 2014; Serracca et al., 2014; Cipriani et al., 2016). However, parasitological surveys concerning the distribution of *Anisakis* spp. in *E. encrasicolus* in a broader range of fishing grounds, in fish of different size and relationships between infection rates and accurate data on the localization of larvae in the host, are rare, in particular with respect to the infection of the flesh.

The aim of the present study was to obtain epidemiological data on *Anisakis* spp. larvae in the viscera and flesh of European anchovies caught in different fishing areas of the Mediterranean Sea in order to: (i) identify the species of *Anisakis* larvae using genetic markers; (ii) provide epidemiological data from each sampling area; and (iii) find correlations between fish biological and ecological data with the infection levels recorded.

Data obtained with this study could have important implications in the esteem of human anisakiasis risk in several Mediterranean countries. Raw consumption of E. encrasicolus is one of the major source of *A. pegreffii* infection in Italy (Mattiucci et al., 2013), in Spain (Bao et al., 2017), and in Croatia (Mladineo et al., 2016).

2. Materials and methods

2.1. Fish sampling

A total of 4152 specimens of European anchovies *E. encrasicolus* were obtained from different fishing areas of the Mediterranean Sea (Fig. 1, Table 1) between June 2013 and June 2016 (Table 1) under the framework of the PARASITE Project. The fish, collected early in the morning at fish landings, were immediately frozen and shipped by a refrigerated truck to the Laboratory of Parasitology at the Department of Public Health and Infection Diseases of "Sapienza-University in Rome" and to the Laboratory for Aquaculture of the Institute of Oceanography and Fisheries in Split, Croatia. The fish were kept frozen at −20 °C until their parasitological examination. The temperature was monitored during the transport using a data logger, and it was maintained constantly below 0 °C for the refrigerated samples, taking into account that post-mortem migration could occur in fish flesh (Cipriani et al., 2016).

2.2. Parasitological analyses

All 4152 anchovies collected were measured (total length) to the nearest 0.1 cm and weighed before being processed for a parasitological examination. The mean lengths and weights of the different samples of anchovies are reported in Table 1. Fulton's condition factor ($K = W(g) \times 10^5/L^3$ (mm)) was calculated for anchovies from each sampling area.

Fish were washed to remove potentially loose nematodes or those adhering to the external surface of fish body. Thereafter, the water collected was sieved to collect any larvae present. The anchovies were then gutted, and the visceral content of each individual fresh fish was separated. The flesh was cut into butterfly fillets and placed next to the visceral content in individual plastic bags, pressed under hydraulic pump and stored overnight at −20 °C for subsequent UV-based detection of larvae (Karl and Leinemann, 1993; Karl and Levsen, 2011; Levsen et al., submitted for publication). In addition, to monitor any dispersion of migrating

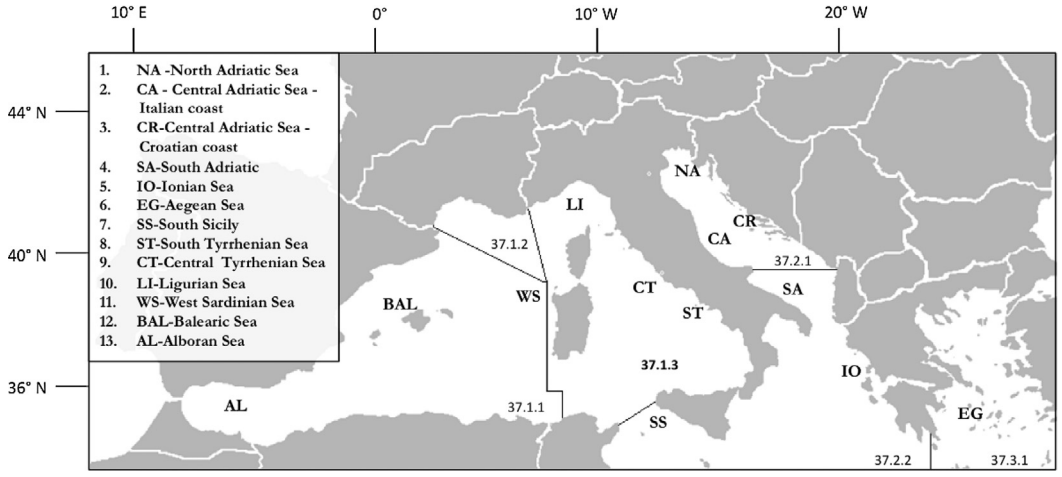

Fig. 1. *Engraulis encrasicolus* sampling localities (also see Table 1).

Table 1
Number (N), total length means and ranges, total weight means and ranges of the anchovies *Engraulis encrasicolus* inspected in the different sampling area here studied. Number (N_{coll}) of total larval nematodes collected, and number (N_{id}) of larvae identified.

	Engraulis encrasicolus			N_{coll} collected larvae	N_{id} identified larvae
	N fish	Mean length ± SD (range)	Mean weight ± SD (range)		
NA – North Adriatic Sea (45°28′ N–13°0′ E)	645	129.12 ± 10.70 (119–150)	14.50 ± 1.24 (8.16–20.44)	2073	46
CA – Central Adriatic Sea–Italian coast (42°58′ N–14°12′ E)	528	138.42 ± 9.84 (120–168)	14.48 ± 2.67 (10–29)	2483	210
CR – Central Adriatic Sea–Croatian coast (43°6′ N–16°21′ E)	518	139.00 ± 5.26 (117–159)	17.00 ± 2.32 (12–29)	1432	171
SA – South Adriatic Sea (41°23′ N–16°50′ E)	120	137.00 ± 7.60 (110–160)	16.75 ± 2.32 (11–23)	340	119
IO – Ionian Sea (37°49′ N–20°16′ E)	160	114.00 ± 6.08 (100–135)	9.49 ± 1.58 (6–15)	0	0
EG – Aegean Sea (37°42′ N–24°34′ E)	108	134.12 ± 9.00 (110–155)	16.18 ± 4.00 (9–27)	2	1
SS – South Sicily (37°28′ N–12°29′ E)	200	130.27 ± 5.54 (120–150)	14.84 ± 1.84 (10–25)	1	1
ST – South Tyrrhenian Sea (40°59′ N–12°59′ E)	554	126.16 ± 10.43 (100–160)	13.63 ± 3.27 (6–25)	9	7
CT – Central Tyrrhenian Sea (42°4′ N–11°43′ E)	336	124.47 ± 12.90 (80–160)	13.20 ± 4.18 (4–30)	9	7
LI – Ligurian Sea (43°57′ N–9°41′ E)	433	137.01 ± 14.63 (100–170)	17.76 ± 5.76 (6–31)	44	31
WS – Sardinian sea (40°28′ N–08°05′ E)	200	138.87 ± 8.55 (120–160)	18.58 ± 3.94 (10–30)	33	24
BAL – Balearic sea (40°23′ N–4°28′ E)	100	130.65 ± 6.10 (120–145)	16.22 ± 2.04 (12–23)	1	1
AL – Alboran Sea (36°34′ N–3°30′ E)	250	127.70 ± 20.28 (90–160)	15.42 ± 9.33 (6–34)	0	0

larvae in the transport boxes, the residual liquid of the stored fish was filtered by a sieve as described above.

Larvae were counted and designated to the corresponding genus using an optical microscope, according to diagnostic morphological keys (Berland, 1961). *Anisakis* spp. and *Hysterothylacium* spp. larvae were washed in saline solution and stored in Eppendorf tubes at −70 °C until further genetic identification.

All the data for the fish (sampling area, date of catch, length, weight) and nematodes (number, localization in the fish host, identification) was collected and reported in a Biobanking platform specifically designed within the PARASITE project (González et al., submitted for publication).

2.3. Genetic identification

Species identification of a subsample of 547 *Anisakis* spp. larvae (equivalent to 17.1% of the total 3202 larvae collected) recovered from fish specimens caught at different fishing areas was done by a multi-marker nuclear genotyping approach. Each specimen of *Anisakis* was cut into two parts: one part was used for scoring three diagnostic allozyme loci, whereas the other part was stored in 96% ethyl alcohol until DNA extraction. Total DNA was extracted using a Quick-gDNA MiniPrep (Zymo Research Corp, CA, USA) from 2 mg of homogenized tissues from each specimen, following the manufacturer's protocol (detailed procedure in Levsen et al., submitted for publication).

Diagnostic allozyme loci (*Adk-2*, *Pep C-1* and *Pep C-2*) were analyzed according to the published procedures (see Mattiucci et al., 1997, 2014) in 547 *Anisakis* spp. larvae. Additionally, nDNA EF1 α-1 and mtDNA *cox2* genes were partially sequenced in a subsample of 141 larvae, randomly selected among those identified by allozymes (Mattiucci et al., 2016). The nuclear gene elongation factor 1-alpha 1 (EF1 α-1) was amplified using the primers EF-F (5′-TCCTCAAGCGTTGTTATCTGTT-3′) and EF-R (5′-AGTTTTGCCACTAGCGGTTCC-3′), according to Mattiucci et al. (2016). PCR conditions were as described in detail by Mattiucci et al. (2016). The sequences obtained for the EF1 α-1 nuclear gene for larval specimens analyzed in the present study were compared with those previously obtained from *A. pegreffii* (KT825684) and *A. simplex* (s. s.) (KT825685) (Mattiucci et al., 2016).

The *cox2* gene encoding mitochondrial cytochrome *c* oxidase subunit II was amplified using the primers 211F (5′-TTT TCT AGT TAT ATA GAT TGR TTY AT-3′) and 210R (5′-CAC CAA CTC TTA AAA TTA TC-3′) (Nadler and Hudspeth, 2000; Valentini et al., 2006) spanning mtDNA nucleotide positions 10,639–11,248, as defined in *Ascaris suum* (GenBank X54253). PCR conditions were as previously described by Mattiucci et al. (2014). The sequences obtained in the present study were compared to the following sequences previously published in GenBank: *A. simplex* (s. s.) (DQ116426), *A. pegreffii* (JQ900761), *A. berlandi* (KC809999), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433) and *A. paggiae* (DQ116434).

Additionally, a subsample of 70 specimens (2.2% out of a total 3225 collected) of *Hysterothylacium* spp. from randomly selected *E. encrasicolus*, which originated from different Mediterranean fishing localities, were identified to the species level by the sequence analysis of the rDNA internal transcribed spacers region (ITS). PCR amplification was performed using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) as reported in Zhu et al. (2000). PCR conditions were as described by Zhu et al. (2000). The sequences obtained were analyzed by GenBank Blast software and aligned previously characterized sequences of the Raphidascarididae family using ClustalX (Thompson et al., 1997).

2.4. Statistical analysis of the epidemiological data

Infection levels of *Anisakis* spp. and *Hysterothylacium* spp. larvae in the anchovies were calculated for each sampling area

and presented as: prevalence (*P*, %) with confidence limits (Clopper–Pearson interval), abundance (*A*) and mean intensity (mI) as described by Bush et al. (1997), Rózsa et al. (2000) and Reiczigel (2003). These values were calculated using web-based Quantitative Parasitology (QPweb) software (Reiczigel and Rózsa, 2005). Statistical significance of differences in the values of prevalence and abundance of *Anisakis* spp. larvae were assessed by the Fisher's exact test and bootstrap *t*-test, respectively, using QPweb. Differences were considered significant when $P < 0.05$.

Subsequently, all sampling data from different areas were pooled, and a set of Generalized Linear Models (GLMs) was implemented to test for the effect of sampling zone (S_a) and fish length (F_L) on parasitic abundance (n). Abundance data were log-transformed ($\log n + 1$) in order to obtain a set of normally distributed values. Four different GLMs were implemented to test for the effects of explanatory variables and their mutual dependence/independence: (a) GLM_1, independence between variables, (b) GLM_2, full dependence between variables, (c) GLM_3, n dependent on F_L and S_a, (d) GLM_4, n dependent on F_L. Models were compared by using the Akaike's information criterion (AIC, Anderson et al., 1998), and results were expressed as AIC differences (ΔAIC), with the best model having $\Delta AIC = 0$.

Correlation between fish length and abundance was tested by means of robust linear regression designed to circumvent some limitations of traditional parametric and non-parametric approaches, when the distribution of errors is asymmetric or prone to outliers.

To test for the relationship between Fulton's *K* and *Anisakis* spp. abundance, GLMs, which could accommodate for various non-normal error distributions of data, were run for the samples obtained from different geographic areas. For parasite abundance in fish, a logistic regression model with a binomial error distribution and logit link function was applied. Thus, the *G* statistic (i.e., χ^2 with one degree of freedom giving significance for the slope) was used to measure the difference in deviance between the full model and an additional GLM run, where only the intercept was fitted.

3. Results

3.1. *Identification of* Anisakis *spp. and* Hysterothylacium *spp. larvae*

Anisakis spp. larvae ($N = 3202$) collected from the examined anchovies were first assigned to larval morphotype Type I, and their relationship to the genus *Anisakis* was determined based on available morphological characters at genus level (Berland, 1961). In addition, 3225 larval nematodes were assigned morphologically (sensu Moravec, 1994) to the genus *Hysterothylacium*.

According to the alleles of the diagnostic loci, i.e., *Adk-2*^{100}, *PepC-1*^{100} and *PepC-2*^{100}, a subsample of *Anisakis* type I larvae ($N = 547$) from all sampled localities were assigned to the species *A. pegreffii*. A subsample of allozyme-identified *A. pegreffii* ($N = 141$) was confirmed also by the partial sequence of the nuclear gene encoding EF1 α-1, according to diagnostic nucleotide positions of that locus (Mattiucci et al., 2016). Six EF1 α-1 nDNA gene sequences obtained in this work from *A. pegreffii* larvae were deposited in GenBank (accession numbers: KY565565, KY565566, KY565567, KY565568, KY565569, KY565570).

Furthermore, sequencing of the mitochondrial *cox2* gene in these same 141 samples of *Anisakis* spp. also identified them as *A. pegreffii* with a 99–100% match to *A. pegreffii* sequences previously deposited in GenBank (Mattiucci et al., 2014). Six sequences of the mitochondrial *cox2* of *A. pegreffii* from *E. encrasicolus* were deposited in GenBank (accession numbers: KY565559, KY565560, KY565561, KY565562, KY565563, KY565564).

In addition to *Anisakis* larvae, 3225 specimens of raphidascaridid nematodes belonging to the genus *Hysterothylacium* were recovered in syntopy with *Anisakis* spp. larvae in the viscera of the anchovies examined. Notably, *Hysterothylacium* worms were never found to infect fish fillet. A subsample of *Hysterothylacium* spp. ($N = 70$), which originated from different fishing areas, was assessed by genetic markers (sequence analysis of rDNA ITS region) to determine their species identity. The sequences obtained (936 bp) matched by 99% with rDNA ITS sequence of *Hysterothylacium aduncum* deposited in GenBank (JQ934878) (Smrzlić et al., 2012). rDNA ITS sequences obtained from *H. aduncum* larvae in this work were deposited in GenBank (accession numbers: KY595228, KY595229).

3.2. *Levels of parasitic infection with* A. pegreffii *larvae*

Data on the prevalence and abundance of *A. pegreffii* larvae at different sites (visceral cavity with internal organs and flesh), and their relative proportions in different fishing grounds of the Mediterranean Sea are given in Table 2.

No significant differences ($P > 0.05$) in the prevalence or abundance of the infection with *A. pegreffii* were found between batches of anchovies fished from the same locality in different seasons of the year. As a consequence, seasonal data were pooled and only fishing area was used as factor.

Rates of *Anisakis pegreffii* occurrence in anchovy samples from different Mediterranean localities showed a significantly uneven distribution ($P < 0.001$; Table 2). In particular, the highest levels of infection with the parasite (prevalence, $P = 70.8$; mean abundance, $A = 4.30$, range 0–45; Table 2) were noted in anchovies caught off the Italian coast in the central area of the Adriatic Sea (sampling area 2 in Fig. 1 and Table 2). High infection levels have also been recorded in the southern area of the Adriatic Sea (prevalence, $P = 55.8$ and mean abundance, $A = 2.62$) and off the Croatian coast in the central area of the Adriatic Sea ($P = 39.8$; $A = 0.98$), which were, respectively, sampling areas 4 and 3 in Fig. 1 and Table 2. In contrast, samples from the northern area of the Adriatic Sea showed considerably lower infection levels, with prevalence $P = 2.8$ and abundance $A = 0.04$ (Table 2). Focusing on samples from different areas of the Adriatic Sea basin, we observed congruency between particular areas. Likewise, infection levels in anchovies from the southern Adriatic and off the Croatian coast of Split showed similar prevalence ($P = 55.8$ and $P = 39.8$, respectively) and abundance ($A = 2.62$ and $A = 0.98$, respectively) values, which were not significantly different from each other ($P > 0.06$). However, heavy rates of infection of samples from the central area of the Adriatic Sea were different ($P < 0.01$) from those in all other studied localities.

No *Anisak*is larvae were detected in the samples of anchovies from the Ionian Sea, waters off Southern Sicily or from the Alboran Sea (Table 2). The samples from the Aegean and Balearic Seas showed low infection levels ($P = 0.01$ and $A = 0.01$); only one *A. pegreffii* larva was found in *E. encrasicolus* samples in each of the two localities (Table 2). Anchovy samples from the southern and central areas of the Tyrrhenian Sea (ST – off Gaeta coast and CT – off Piombino coast, respectively) showed low infection values with no statistically significant differences between them ($P > 0.05$; Table 2). The samples of anchovies fished in the Ligurian Sea and off the North West coast of Sardinia were not significantly different in pairwise comparisons ($P > 0.05$) and exhibited similar values for both the prevalence and abundance values of infection (7.5% vs. 11.5% and 0.09 vs. 0.15, respectively; Table 2).

After pooling all fish samples, the relative proportion of *A. pegreffii* larvae infecting the viscera of the anchovies was 95.5% ($N = 3057$), whereas the relative proportion of the parasites detected in fish flesh was 4.5% ($N = 145$). When different samples of anchovies were compared in pairs, no significant differences were observed between relative frequencies of *A. pegreffii* in the viscera and fish

Table 2
Prevalence (*P*, %), mean abundance (*A*) and mean intensity (mI and its range) values of *Anisakis pegreffii* in *E. encrasicolus* collected in each sampling areas. Number of total larvae (N_{Tot}) and their relative proportions (%) in different tissues are also given.

	N fish	Viscera					Flesh					Total				
		P (%)	*A*	mI	Range	Tot (%)	*P* (%)	*A*	mI	Range	Tot (%)	*P* (%)	*A*	mI	Range	Tot
NA	645	2.8 0.017–0.04	0.039 ± 0.27	1.39 ± 0.92	(0–4)	25 (100.0%)	0	0	0	0	0	2.8 0.017–0.04	0.039 ± 0.27	1.39 ± 0.92	(0–4)	25
CA	528	69.5 0.65–0.73	4.12 ± 6.04	5.93 ± 6.46	(0–45)	2178 (96.0%)	14.6 0.12–0.18	0.18 ± 0.48	1.23 ± 0.51	(0–3)	95 (4.0%)	70.8 0.67–0.75	4.30 ± 6.22	6.08 ± 6.63	(0–45)	2273
CR	518	38.4 0.34–0.43	0.92 ± 1.90	2.40 ± 2.43	(0–15)	477 (94.5%)	4.8 0.03–0.07	0.054 ± 0.25	1.12 ± 0.33	(0–2)	28 (5.5%)	39.8 0.35–0.44	0.98 ± 1.98	2.45 ± 2.49	(0–17)	505
SA	120	53.3 0.44–0.62	2.47 ± 4.61	4.64 ± 5.48	(0–25)	297 (94.6%)	12.5 0.07–0.20	0.14 ± 0.39	1.13 ± 0.35	(0–2)	17 (5.4%)	55.8 0.46–0.65	2.62 ± 4.75	4.69 ± 5.55	(0–25)	314
IO	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EG	108	0.009 0–0.05	0.009 ± 0.096	1 ± 0.00	(0–1)	1 (100.0%)	0	0	0	0	0	0.009 0–0.05	0.009 ± 0.096	1 ± 0.00	(0–1)	1
SS	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ST	554	1.4 0.006–0.028	0.014 ± 0.72	1 ± 0.00	(0–1)	8 (89.9%)	0.2 0.00–0.01	0.002 ± 0.04	1 ± 0.00	(0–1)	1 (11.1%)	1.6 0.007–0.03	0.016 ± 0.13	1 ± 0.00	(0–1)	9
CT	336	2.4	0.024 ± 0.15	1 ± 0.00	(0–1)	8 (100.0%)	0	0	0	0	0	2.4	0.024 ± 0.15	1 ± 0.00	(0–1)	8
LI	433	7.2 0.05–0.10	0.08 ± 0.31	1.13 ± 0.43	(0–3)	35 (94.6%)	0.5 0.001–0.02	0.005 ± 0.07	1 ± 0.00	(0–1)	2 (5.4%)	7.6 0.05–0.11	0.09 ± 0.32	1.12 ± 0.41	(0–3)	37
WS	200	11.5 0.07–0.17	0.14 ± 0.40	1.17 ± 0.39	(0 –2)	27 (93.1%)	1.00 0.001–0.04	0.01 ± 0.1	1.00 ± 0.00	(0–1)	2 (6.9%)	11.5 0.07–0.17	0.15 ± 0.43	1.26 ± 0.45	(0–2)	29
BAL	100	0.10 0.00–0.05	0.100.00–0.05	1 ± 0.00	(0–1)	1 (100.0%)	0	0	0	0	0	0.10 0.00–0.05	0.100.00–0.05	1 ± 0.00	(0–1)	1
AL	250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

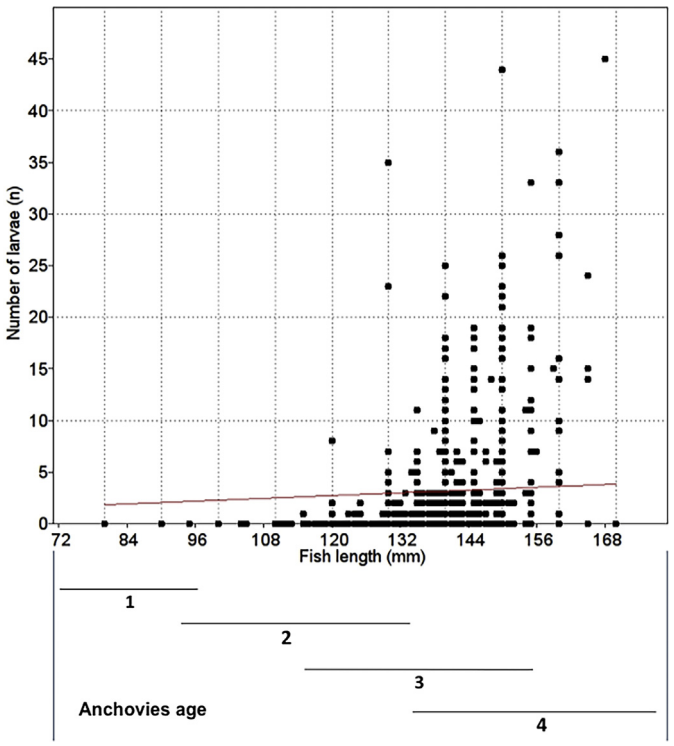

Fig. 2. Analysis of the Spearman's rank-order correlation ($r^2 = 0.08$, $P < 0.01$) between fish length (L) and number of parasites (n). Below, estimate of fish age based on fish length, according to Basilone et al. (2004).

flesh ($P > 0.05$, Yates corrected χ^2 test). The percentage of larvae infecting anchovy flesh ranged from 4.0% to 6.9% in samples from different areas (Table 2). The values of the frequencies observed in different samples (Table 2) showed no statistically significant differences from the frequencies observed when all fish samples were pooled together (see above) (Yates corrected χ^2 test).

When all samples of anchovies were taken into account, the best model for explaining the parasitic burden by *A. pegreffii* in *E. encrasicolus* was GLM_3. This model revealed a relationship between the density of parasites on one hand, and sampling area (S_a) and fish length (F_L) on the other hand. Indeed, differences in length have been found between fish sampled in different geographic areas (Student's t-test, $t = 629.49$, $P < 0.001$). Spearman correlation analysis revealed a general significant correlation ($r^2 = 0.08$, $P < 0.01$) between fish length (F_L) and the number of parasites per fish (n), indicating that bigger fish tend to host higher parasite burdens (Fig. 2).

According to the predictions of the GLM_3 model, the main variables associated with *A. pegreffii* parasite burden were fishing ground and fish length. However, in some fishing areas, smaller-sized *E. encrasicolus* (<130 mm) showed higher levels of infection (samples from the Central and South Adriatic, sampling areas 2–4 in Fig. 1), whereas other samples, characterized by a larger mean size (e.g., 134.12 and 130.65 mm for anchovies from the Aegean and Balearic Seas, respectively), were completely uninfected by *A. pegreffii*.

Fulton's K parameter, related to the body condition index (BCI) of the fish, did not show any significant correlation to abundance of *A. pegreffii* infection ($P > 0.05$) in any of the sampling areas.

3.3. *Levels of parasitic infection with* H. aduncum *larvae*

The levels of infection with *H. aduncum* larvae (Table 3) were significantly different ($P < 0.001$) in anchovies caught at different Mediterranean localities. Anchovies from the Adriatic Sea (sampling areas 1–4 in Fig. 1 and Table 3) exhibited the highest prevalence and mean abundance values of infection with *H. aduncum*. All other samples of *E. encrasicolus* showed lower levels of infection with this parasite. No *H. aduncum* larvae were detected in the flesh of examined fish.

4. Discussion

In the present study, we detected nematode infection levels by using the UV-press standardized methodology (Gómez-Morales et al., 2017; Levsen et al., submitted for publication) in all tissues of a large sample of anchovies ($N = 4152$). Two larval ascaridoids, *A. pegreffii* and *H. aduncum*, were identified in European anchovies from several selected localities in the Mediterranean region. A multi-locus genetic approach based on the study of three diagnostic allozyme loci and the analyses of nuclear EF1 α-1 DNA and mitochondrial *cox2* DNA markers allowed to identify larval subsamples collected from anchovies fished from the Adriatic, Tyrrhenian and Ligurian Seas, and the waters off the North West coast of Sardinia, as *A. pegreffii* (Fig. 1). The sequence analysis of rDNA ITS region allowed the identification of 70 *Hysterothylacium* spp. larvae from the same sampling localities (e.g., Adriatic and Ligurian Sea, Fig. 1).

Anisakis pegreffii occurred in both the viscera and flesh of the anchovies, whereas *H. aduncum* was found to infect only the viscera of *E. encrasicolus*, as previously reported by Cipriani et al. (2016). Out of the two ascaridoid species, only *A. pegreffii* is a zoonotic pathogen, because it is responsible for gastric, intestinal and gastro-allergic anisakiasis in humans (Mattiucci et al., 2011, 2013; Mladineo et al., 2016). On the contrary, *H. aduncum* has not been recognized yet as being pathogenic for humans. Furthermore, considering that its larvae do not migrate into the flesh of fish host, *H. aduncum* probably does not have a direct effect on food safety (Levsen and Karl, 2014).

In addition, *E. encrasicolus* has been reported as the main source of human cases of anisakiasis in Italy (Moschella et al., 2004; Mattiucci et al., 2011, 2013). Infection with live and pathogenic larvae occurs usually after the consumption of flesh fillets of raw homemade marinated anchovies (Mattiucci et al., 2011, 2013). Consequently, epidemiological data about rates of infection of the target fish host flesh by *A. pegreffii* larvae in different areas of the Mediterranean basin represents a crucial dataset for evaluating the risk to humans associated to this parasite species.

Additionally, all fish samples examined in this study were frozen within one hour after fish landing to prevent *post mortem* migration of *A. pegreffii* larvae, a temperature- and time-dependent event that can occur during the storage of this fish species (Šimat et al., 2015; Cipriani et al., 2016). The results confirmed the occurrence of *A. pegreffii* larvae in the flesh of *E. encrasicolus* as a result of *intra-vitam* migration, as previously observed (Cipriani et al., 2016). The overall relative proportion of *A. pegreffii* larvae located in fish flesh was 4.2% of the total number of larvae collected, which was much lower than the frequency of 95.8% with which the parasites were detected in the visceral cavity and/or embedded in the visceral organs of the fish host (Table 2). Similarly, rare occurrence of *A. pegreffii* in fish flesh (4.0%) was observed in our previous study (Cipriani et al., 2016).

GLM_3 model built on the basis of observed infection data revealed a significant correlation between fish size and *A. pegreffii* burden (Fig. 2). This correlation is most probably explained by a direct relationship between the size of the fish and its age (Fig. 2), as reported by Basilone et al. (2004). In the pooled sample of *E. encrasicolus*, it emerged that fish specimens that were likely 3–4 years of age (range: 135–168 mm long) tended to have a higher *A. pegreffii* burden (Fig. 2). The age of the fish has a direct relation to the

Table 3
Prevalence (*P*, %), mean abundance (*A*) and mean intensity (mI and its range) values of infection with *Hysterothylacium aduncum* larvae identified in *E. encrasicolus* recorded in the sampling areas.

	P (%)	*A*	mI	Range	Tot larvae
NA – North Adriatic Sea	15.5	3.18 ± 5.15	6.17 ± 5.75	(1–34)	2048
CA – Central Adriatic Sea–Italian coast	24.8	0.40 ± 0.80	1.60 ± 0.79	(1–4)	210
CR – Central Adriatic Sea–Croatian coast	73.4	1.79 ± 1.70	2.44 ± 1.54	(1–10)	927
SA – South Adriatic Sea	15.0	0.21 ± 0.61	1.44 ± 0.85	(1–4)	26
IO – Ionian Sea	0.0	0.00 ± 0.00	0.00 ± 0.00	(0–0)	0
EG – Aegean Sea	0.9	0.01 ± 0.01	1.00 ± 0.00	(0–1)	1
SS – South Sicily	0.5	0.01 ± 0.07	1.00 ± 0.00	(0–1)	1
ST – South Tyrrhenian Sea	0.0	0.00 ± 0.00	0.00 ± 0.00	(0–0)	0
CT – Central Tyrrhenian Sea	0.3	0.01 ± 0.05	1.00 ± 0.00	(0–1)	1
LI – Ligurian Sea	1.2	0.02 ± 0.16	1.40 ± 0.55	(1–2)	7
WS – Sardinian sea	2.0	0.02 ± 0.14	1.00 ± 0.00	(1–1)	4
BAL – Balearic Sea	0.0	0.00 ± 0.00	0.00 ± 0.00	(0–0)	0
AL – Alboran Sea	0.0	0.00 ± 0.00	0.00 ± 0.00	(0–0)	0

foraging time on the zooplanktonic organisms that serve as hosts of *A. pegreffii* larvae. Considering that *A. pegreffii* larvae accumulate in the fish host and probably stay for an undetermined period, the correlation between the size of the fish and parasite burden is a direct consequence of the time the fish had spent feeding on infected invertebrate organisms, which, in turn, positively correlates with the age of the fish. According to Spearman's rank correlation analysis, no statistically significant correlation was found between the Fulton's condition factor *K* and *A. pegreffii* abundance ($P > 0.05$). Both fish from heavily infected stocks and fish that were not infected maintained a constant proportion of weight and length, showing similar BCI values. This seems to suggest that *Anisakis* spp. larvae do not have a pathogenic role in this fish species.

The levels of parasitic infection with *A. pegreffii* recorded in the *E. encrasicolus* samples from different fishing grounds throughout the Mediterranean Sea were heterogeneous as a consequence of two drivers of the infection: the fishing ground and fish size (Fig. 2). Thus, even though a high significant correlation between fish size and *A. pegreffii* burden was detected in all samples (Fig. 2), the fishing ground seemed to be the main factor that affected the density of this parasite species. As a result, the geographical location could possibly predict the presence of the parasite in randomly collected anchovy samples (Table 2). Among all fish sampling localities considered, anchovies from the central area of the Adriatic Sea basin showed the highest levels of infection by *A. pegreffii*, with the highest parasite burden recorded off the Italian coast. In addition, high levels of infection with the same *Anisakis* species have been recorded at the same latitude in this study (Table 2) and, previously, off the Croatian coast (Mladineo et al., 2012). In the anchovies fished from the central Adriatic Sea, the highest amount of larvae per single infected fish was recorded in a 168-mm long specimen that harboured 45 larvae, all located in the viscera.

With regard to the sampling localities in the western Mediterranean Sea (Table 4), fish from the Ligurian Sea showed high levels of infection by *A. pegreffii* ($P = 7.5\%$; $A = 0.09$; Table 2), in accordance with previously reported data by Rello et al. (2009). This finding could be related to an abundant fauna of dolphins and whales – definitive hosts of the parasite species (Mattiucci and Nascetti, 2008; Mattiucci et al., 2014), inhabiting this part of the Mediterranean Sea, which hosts the Ligurian Sea Cetacean Sanctuary.

Thus, the eastern Mediterranean region and, in particular, the central Adriatic Sea area, represents a "hotspot" for the presence of *A. pegreffii* in anchovies, showing the highest infection levels in the Mediterranean Sea (Table 2). This finding could be related to ecological characteristics of this basin, with both abiotic and biotic conditions sustaining *A. pegreffii* life cycle with high population density.

The Adriatic Sea basin represents a peculiar area within the Mediterranean Sea. The northern section of the basin is characterized by very shallow and gently sloping waters, whereas the central area of the Adriatic Sea, having an average depth of 140 m, shows some open sea characteristics (Russo and Artegiani, 1996). In contrast, its southern area has steep slopes, higher salinity and a maximum depth of 1200 m (Cushman-Roisin et al., 2001), and can be considered as a pelagic oceanic habitat (Fonda-Umani, 1996). Circulation patterns in the Adriatic are generally cyclonic: a northward current flows along the eastern coast, whereas a southward current flows along the western coast (Russo and Artegiani, 1996). Each of the three sub-basins exhibits, with a certain seasonal variability, cyclonic gyres. This gyre system maintains the basin as a peculiar ecosystem with a slow exchange of planktonic organisms with the rest of the Mediterranean Sea (Koblmüller et al., 2015). Nutrient levels in the northern Adriatic are clearly controlled by several river inputs that induce intense phytoplankton development in winter and autumn (Zavatarelli et al., 1997). Unlike other sub-basins, the central area of the Adriatic Sea seems to have a constant nutrient supply partly due to the proximity to freshwater river inputs, and partly due to cyclonic gyres, which provoke a constant upwelling of deep, nutrient-rich waters to the surface. Both conditions seem to have a direct impact on the presence, abundance and distribution of abundant biomass of planktonic organisms. The latter organisms comprise first intermediate hosts of *A. pegreffii*. In turn, high biomass of planktonic organisms supports populations of small pelagic plankton feeder fish, such as anchovies and sardines, which act as secondary intermediate/paratenic host for this nematode. Furthermore, both water circulation and the intensity of primary production have been cited as factors regulating the intensity of anchovy spawning in this particular area of the Adriatic Sea (Regner, 1996). Thus, mean sea surface temperature, depth, salinity, as well as primary production, were identified as most important abiotic variables affecting the distribution of *Anisakis* spp. (Højgaard, 1998; Kuhn et al., 2016). At higher trophic level, the northern and central areas of the basin are inhabited by several species of cetaceans, mainly represented by bottlenose dolphins (*Tursiops truncatus*) and, rarely, by striped dolphins (*Stenella coeruleoalba*). Populations of bottlenose dolphin may represent a suitable host for *A. pegreffii* in that area of the Adriatic Sea, because they prey upon anchovies, hakes, and other important commercial fish species (Holcer et al., 2014). Blažeković et al. (2015) reported high levels of *A. pegreffii* abundance in bottlenose dolphins stranded along the Croatian coast. Studies of this dolphin species revealed a fine-scale genetic structure of its populations throughout the Adriatic Sea inferred by nuclear DNA markers, showing a differentiation between populations in the Northern and Central-South sub-basins, as well as between West and East coasts (Gaspari et al., 2015). This suggests that local populations of *T. truncatus* tend to persist in different areas of the basin, feeding on populations of prey items of "local" food webs.

Table 4
Historical data from recent scientific literature on *A. pegreffii* and *H. aduncum* infection levels recorded in the same localities of the Mediterranean Sea here understudied. Year of sampling, number of fish examined (N_{fish}) with mean fish length, prevalence (*P*) and abundance (*A*) are reported.

Fishing area	Year of sampling	N_{fish} mean length (mm)	*Anisakis* spp.		*Hysteroyhylacium* spp.		Author
			P	*A*	*P*	*A*	
North Adriatic Sea	2015	4350	0.005	0.006	0.28	0.287	Cavallero et al., 2015
Central Adriatic Sea (Croatian coast)	2009–2011	2300 (165 mm)	81.1	7.58	–	–	Mladineo et al., 2012
Aegean Sea	2009	462 (124 mm)	3.90	0.05	–	–	Chaligiannis et al., 2012
South Tyrrhenian Sea	2012	490 (92 mm)	0.00	–	0.00	–	De Liberato et al., 2013
Central Tyrrhenian Sea	2012	500 (102 mm)	1.40	–	1.60	–	De Liberato et al., 2013
Ligurian Sea	2012	1050 (110 mm)	0.80	0.01	–	–	Serracca et al., 2014
	1998–1999	64 (133 mm)	21.88	0.78	70.31	4.03	Rello et al., 2009
Sardinian Sea	2006–2011	38 (149 mm)	65.80	1.84	–	–	Piras et al., 2014
	2008–2010	52	34.60	1.25	67.30	2.40	Angelucci et al., 2011
Gulf of Lion	1998–1999	103 (133 mm)	3.88	0.06	68.93	11.74	Rello et al., 2009
Alboran Sea	1998–1999	72 (129 mm)	1.40	0.01	2.80	0.11	Rello et al., 2009

Abiotic conditions of the central Adriatic Sea favour the presence of a stable food web and may enhance the overlapped and abundant distribution of hosts belonging to different trophic levels of the life cycle of *A. pegreffii*. This circumstance could explain the pattern of *A. pegreffii* distribution and high infection levels in this semi-enclosed and narrow basin. In other words, both abiotic (water circulation) and biotic factors could represent a direct advantage for the persistence of the parasite life cycle in that Adriatic Sea basin.

Furthermore, another reason for high density of *A. pegreffii* in this area could be the presence of an "anthropogenic shortcut" in the life cycle of the parasite. There is a widely used routine followed by local fishermen that involves eviscerating fish directly on board of the vessels, and discarding these viscera at sea along with by-catch. This practice seems to be related to the need to remove the viscera of fish species frequently infected by *Anisakis* larvae, whose presence would compromise the value of the fish at landing. In the course of this research, we had collected clues that the viscera discards, originating from various commercial fish species of that area, have been dropped at sea after fishing activities along with common by-catch discards. Those viscera discards, including still alive *Anisakis* larvae, once at sea, may be consumed by both fish and cetaceans organisms, which would consequently acquire *Anisakis* larvae. The phenomenon of increased parasite transmission due to attraction of piscivorous birds and marine mammals, i.e., final hosts, to discards from fishing has been previously described for other parasites life cycle (Oro and Ruiz, 1997; Morton and Yuen, 2000; Arcos et al., 2001; Bozzano and Sardà, 2002). Additionally, as it happens in other areas around the world, in this area, cetaceans have "learned" to follow bottom trawlers to take advantage of fish caught, stirred up or attracted by the net, or discarded from the nets after trawling (e.g., Leatherwood, 1975; Corkeron et al., 1990; Waring et al., 1990; Morizur et al., 1999; Goffman et al., 2001). Large populations of *T. truncatus* are present in that geographical area of the Mediterranean Sea and this species is most frequently involved in interactions with coastal fisheries. We have collected several observations and reports by local fishermen about constant presence of *T. truncatus* following trawlers during their activity at sea, eventually feeding on the discards dropped from the nets and deck.

We thus hypothesize that dolphins directly feeding on these discarded viscera could acquire large numbers of *Anisakis* larvae, due to an "anthropogenic shortcut" in the parasite life cycle. This, in turn, maintains a high level of parasite density in that area of the Adriatic Sea. This anthropogenic shortcut could involve not only the final hosts of *A. pegreffii*, but also enhance the presence of the parasite in other fish species important for fishermen. Other commercial fish species feeding on the discards and fuelling this "dog chasing its tail" cycle could also be one of the reasons for high levels of infection with *A. pegreffii* in this area of the Adriatic basin.

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