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The Mediterranean European hake, *Merluccius merluccius*: Detecting drivers influencing the *Anisakis* spp. larvae distribution

Paolo Cipriani^{a,b,*}, Gian Luca Sbaraglia^b, Michela Paoletti^b, Lucilla Giulietti^b, Bruno Bellisario^b, Marialetizia Palomba^b, Ivana Bušelić^c, Ivona Mladineo^c, 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

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ABSTRACT

The European hake *Merluccius merluccius* is one of the most commercially important and widely distributed fish species, occurring both in European and Mediterranean Sea fisheries. We analyzed the distribution and infection rates of different species of *Anisakis* in *M. merluccius* (N = 1130 hakes), by site of infection in the fish host (viscera, dorsal and ventral fillets) from 13 different fishing grounds of the Mediterranean Sea (FAO area 37). The fillets were examined using the UV-Press method. A large number of *Anisakis* specimens (N = 877) were identified by diagnostic allozymes, sequence analysis of the partial EF1 α -1 region of nDNA and mtDNA *cox2* gene. Among these, 813 larvae corresponded to *A. pegreffii*, 62 to *A. physeteris*, 1 to *A. simplex* (s. s.), whereas one resulted as a F1 hybrid between *A. pegreffii* and *A. simplex* (s. s.). Remarkably high levels of infection with *A. pegreffii* were recorded in hakes from the Adriatic/Ionian Sea compared to the fish of similar length obtained from the western Mediterranean fishing grounds. A positive correlation between fish length and abundance of *A. pegreffii* was observed. Concerning the localization of *A. pegreffii* larvae in the fish, 28.3% were detected in the liver, 62.9% in the rest of the viscera, 6.6% in the ventral part of the flesh, whereas 2.1% in the dorsal flesh.

1. Introduction

The European hake *Merluccius merluccius* (L., 1758) is one of the most economically important fish species, occurring both in North-East Atlantic (Casey and Pereiro, 1995) and Mediterranean Sea fisheries (Oliver and Massutí, 1995; Ardizzone and Corsi, 1997; Abaunza et al., 2001). The existence of different intra-specific stocks in European waters has been suggested on the basis of a multi-method approach (Inada, 1981; Alheit and Pitcher, 1995; Roldan et al., 1998; Lundy et al., 1999; Mattiucci et al., 2014a). In fact, north-east Atlantic and Mediterranean Sea populations have been considered as genetically separated stocks (Abaunza et al., 2001; Cimmaruta et al., 2005; Milano et al., 2014). The Atlantic and Mediterranean hake populations exhibit remarkable differences in demographic and life history traits, such as growth rate, size at maturity, recruitment patterns and spawning season (Froese and Pauly, 2013). The species is caught commercially throughout its range. It has a high price in Europe, being mainly sold fresh in both Italian and Spanish markets, and represents one of the most valuable and heavily exploited demersal species in the western European fisheries (Casey and

Pereiro, 1995).

The genus *Anisakis* includes species of heteroxenous parasites of marine organisms, with crustaceans as first intermediate hosts, fishes and squids as intermediate and/or paratenic hosts, and mainly cetaceans as definitive hosts (Mattiucci and Nascetti, 2006, 2008). The larval stages of *Anisakis* spp. commonly infect the viscera and musculature of many teleost species (Mattiucci and Nascetti, 2008; Mattiucci et al., 2017a). Nine species of *Anisakis* have been detected and genetically characterized (Mattiucci et al., 2009; 2014b). Their third larval stage, recovered from fish hosts and invertebrates, exhibits morphotypes indicated as *Anisakis* Type I or Type II (*sensu* Berland, 1961), but they cannot be identified at the species level based on conventional morphological analysis. Among the genetic methodologies used for the species identification of *Anisakis* larvae, allozyme markers represent a valuable tool. Allozymes markers are based on several diagnostic loci, that being fixed for alternative alleles among those highly polymorphic loci studied in distinct species of *Anisakis* (see Mattiucci et al., 2009), permit to recognize the species at any life-history stages. In addition, a new nuclear marker based on the sequences analysis of the EF1 α -1

* 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).

ndDNA locus has been found. It allows the correct identification of the two species *A. simplex* (s. s.) and *A. pegreffii*, due to fixed alternative nucleotide positions in the two species (Mattiucci et al., 2016). Further, the mtDNA *cox2* revealed fixed alternative nucleotide differences in distinct species of the genus *Anisakis*, which allows their correct identification (Valentini et al., 2006). The combined use of those different markers permits a multilocus genotyping approach in the identification process of *Anisakis* spp. specimens (Mattiucci et al., 2014b, 2016).

Previous genetic/molecular identification of *Anisakis* spp. larvae occurring in the European hake *M. merluccius* sampled in the Mediterranean Sea revealed that *A. pegreffii* and *A. physeteris* are the most common species, whereas *A. simplex* (s. s.) and *A. typica* were recovered, respectively, only in Southern areas or nearby Gibraltar Strait and the Alboran Sea (Mattiucci et al., 2004; Valero et al., 2006; Farjallah et al., 2008). In addition, statistically significant differences observed in the distribution patterns of genetically identified *Anisakis* spp. larvae from hakes caught in different fishing grounds, have also been used as biomarkers in the stock characterization of fish species in European waters (Mattiucci et al., 2004, 2014a). However, no detailed information was available regarding the levels of infection with different species of *Anisakis* by location in the fish host (i.e. viscera and flesh of hake) and from different fishing grounds of the Mediterranean Sea. Indeed, in addition to the ecological aspects related to the differential distribution of *Anisakis* spp. from this fish host, data on their distribution in the edible parts of the fish for humans, as compared with the viscera, are important, since larval *Anisakis* are etiological agents of human anisakiasis. Over the last 30 years, there has been an increase in cases of human anisakiasis throughout the world (Bao et al., 2017; Mattiucci et al., 2017a). So far, among the nine species of *Anisakis* genetically characterized (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009), *A. simplex* (s. s.) and *A. pegreffii* are currently reported as zoonotic species causing human anisakiasis (D'Amelio et al., 1999; Umehara et al., 2007; Fumarola et al., 2009; Mattiucci et al., 2011, 2013, 2017b; Arai et al., 2014; Lim et al., 2015; Mladineo et al., 2016). In addition to the consumer health implications, anisakid nematodes also impact fish quality in terms of aesthetics, due to their repellent appearance when they occur in fish intended for human consumption (Karl and Levsen, 2011; Levsen and Karl, 2014).

The aim of the present study was to detect the major drivers influencing the distribution with *Anisakis* spp. larvae in *Merluccius merluccius* from the Mediterranean Sea. We studied the infection with of *Anisakis* species in the viscera and flesh of European hakes, caught in different fishing grounds of the Mediterranean Sea in order to: i) identify *Anisakis* larvae at species level using multilocus genotyping approach, ii) provide parasite epidemiological data by fish size and fishing grounds for future development of risk assessment models of anisakiasis in the Mediterranean Sea.

2. Materials and methods

2.1. Fish sampling

A total of 1130 specimens of European hake *Merluccius merluccius* were obtained from 13 different fishing grounds of the Mediterranean Sea (Fig. 1 Table 1), between June 2013 and June 2016 (Table 1) in the framework of the PARASITE Project. The fish were collected in the early morning at fish landings, immediately frozen and shipped by a refrigerated truck to the laboratories of the Parasitology Section at the Department of Public Health and Infection Diseases of “Sapienza-University” of Rome, and the Laboratory for Aquaculture of the Institute of Oceanography and Fisheries in Split, Croatia. The fish were kept frozen at -20°C until the parasitological examination. The temperature of storage was monitored during the transport using a data logger, and it was kept constant below 0°C for the refrigerated samples, taking into account that a *post-mortem* migration of the *Anisakis* spp. larvae present could occur in the fish flesh when storage temperature is above 2°C

(Cipriani et al., 2016).

2.2. Parasitological analysis

All the specimens were measured (total length) to the nearest 0.1 cm and weighted before being processed for the parasitological examination. The mean length and weight are reported in Table 1. Fulton's condition factor ($K = W(g) \cdot 10^5 / L^3(\text{mm})$) was calculated for hakes of each sampling area.

The hakes were gutted, and the viscera of each individual was separated. Flesh was cut into two to four portions of fillets, separating the hypaxial (ventral) and epaxial (dorsal) regions, following the horizontal septum, and afterwards it was placed in individual plastic bags, pressed under hydraulic press and stored overnight at -20°C for subsequent detection of larvae under UV light (Karl and Leinemann, 1993; Karl and Levsen, 2011; Levsen et al., in press). The larvae were counted and identified to genus level using an optical microscope according to the diagnostic morphological keys (*sensu* Berland, 1961). *Anisakis* spp. Types I and II, and *Hysterothylacium* spp. larvae were washed in saline solution and stored in Eppendorf tubes at -70°C until their 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., 2017).

2.3. Genetic identification of larval nematodes

A subsample of 877 *Anisakis* spp. larvae (out of the total 28783 collected), corresponding to 3% of the larvae recovered in all the fish specimens from different fishing grounds, were identified to the species level by a multi-marker genotyping approach. Each specimen of *Anisakis* was cut in two parts; one part was used for three diagnostic allozymes loci (Mattiucci et al., 2009), whereas the other part was stored in 96% ethyl alcohol until DNA extraction. The total DNA was extracted using the Quick-gDNA MiniPrep (column format) by Zymo Research from 2 mg of homogenized tissues from each specimen, following the manufacturer's protocol (Levsen et al., in press).

The diagnostic allozyme loci (*Adk-2*, *Pep C-1* and *Pep C-2*) were analyzed according to established procedures (see Mattiucci et al., 2009, 2014b) on 877 *Anisakis* spp. larvae. In addition, a subsample of 240 larvae randomly selected among those identified by allozymes were studied by direct sequence analysis of mitochondrial (mtDNA *cox2*, 629 bp) (Mattiucci et al., 2014b) and nuclear (elongation factor EF1 α -1 of nDNA, 409 bp) genes (Mattiucci et al., 2016).

The mitochondrial cytochrome C oxidase subunit II (*cox2*) gene was amplified using the primers 211F (5'-TTTTCTAGTTATATAGATTGRTTYAT-3') and 210R (5'-CACCAACTCTTAAATTA TC-3') (Valentini et al., 2006; Mattiucci et al., 2014b). Polymerase chain reaction (PCR) was carried out according to the previously described procedures (Mattiucci et al., 2014b). The sequences obtained at the mtDNA *cox2* for the larval nematodes were compared with those already obtained for the same gene and deposited 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).

The elongation factor (EF1 α -1 nDNA) nuclear gene was amplified using the primers EF-F (5'-TCCTCAAGCGTTGTTATCTGTT-3') and EF-R (5'-AGTTTTGCCACTAGCGGTTCC-3') (Mattiucci et al., 2016). The PCR conditions and procedures followed those reported in Mattiucci et al., 2016. The sequences obtained at the EF1 α -1 gene of the nDNA for the larval specimens of *A. pegreffii* and *A. simplex* (s. s.) were compared at the diagnostic positions (i.e 186, and 286) as previously detailed (Mattiucci et al., 2016).

In addition, 16 larval specimens of *Hysterothylacium* spp. randomly

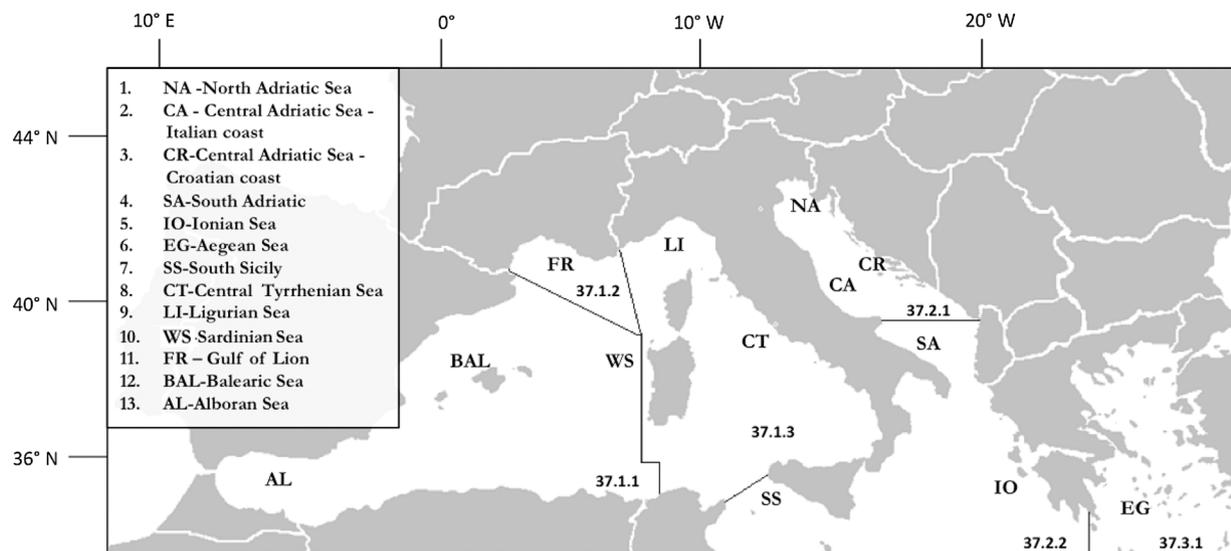


Fig. 1. Sampling localities (see Table 1) of specimens of *Merluccius merluccius*, examined in the present study.

selected from all the infected hakes sampled in the different Mediterranean fishing localities were identified to species level by sequence analysis of the internal transcribed spacers (ITS rDNA) region. The DNA was extracted using the same procedure reported above for *Anisakis* spp. PCR amplification was performed using the primers NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTCTTTCTCCGCT-3'), as reported in Zhu et al. (2000). PCR conditions were those described by Zhu et al. (2000). All the sequences obtained were analyzed by GenBank Blast software and aligned with previously characterized sequences of the Raphidascarididae, by ClustalX (Thompson et al., 1997).

A Bayesian inference (BI) tree, based on mtDNA *cox2* gene sequences of *A. pegreffii* larvae from *Merluccius merluccius* sampled in the Mediterranean Sea, with respect to the other *Anisakis* spp. previously sequenced, was elaborated. The analysis was performed by MrBayes3.1

(Huelsenbeck and Ronquist, 2005) using TIM1 + I + G substitution model as implemented in jModeltest2.1 (Darriba et al., 2012). The parameters for the selected model were I = 0.392 and G = 0.463, calculated with Akaike Information Criterion (AIC) (Posada and Buckley, 2004). For the Bayesian analysis, four incrementally heated Markov Chains (using default heating values) were run for 1,000,000 generations, sampling the Markov Chains at intervals of 100 generations. Numbers at the nodes are posterior probabilities; *Toxocara canis* and *Pseudoterranova ceticola* were used as outgroups.

2.4. Statistical analysis of the epidemiological data

The levels of infection with *Anisakis* spp. larvae in the hakes were calculated for each sampling area. The epidemiological parameters considered were: prevalence (P, %) with confidence limits (Clopper-

Table 1

Collecting localities, number of fish examined (N), mean length, and mean weight of European hake *Merluccius merluccius* analyzed in the present study. Total number of larvae collected per area, and number of larvae genetically identified are reported too.

	N fish	Mean length ± SD (Range)	Mean weight ± SD (Range)	N _{coll} collected larvae	N _{id} identified larvae
NA – North Adriatic Sea (45°28'N – 13°0'E)	73	257.55 ± 12.94 (230–287)	115.13 ± 17.75 (81–176)	669	50
CA – Central Adriatic Sea – Italian coast (42°58'N – 14°12'E)	137	311.45 ± 74.44 (230–640)	245.20 ± 256.52 (87–1800)	13562	130
CR – Central Adriatic Sea – Croatian coast (43°6'N – 16°21'E)	257	212.00 ± 62.46 (130–417)	82.00 ± 92.17 (12–598)	4081	100
SA – South Adriatic Sea (41°23'N – 16°50'E)	46	415.97 ± 74.59 (270–570)	532.17 ± 271.35 (128–1236)	7237	81
IO – Ionian Sea (37°49'N – 20°16'E)	50	470.20 ± 124.12 (315–370)	1002.04 ± 797.13 (245–3200)	2045	75
EG – Aegean Sea (37°42'N – 24°34'E)	27	330.00 ± 18.86 (280–360)	286.78 ± 47.55 (188–373)	69	30
SS – South Sicily (37°78'N – 12°29'E)	64	286.73 ± 47.17 (240–460)	193.87 ± 128.22 (100–765)	412	80
CT – Central Tyrrhenian Sea (42°4'N – 11°43' E)	165	314.34 ± 40.26 (255–470)	239.35 ± 95.79 (134–631)	230	100
LI – Ligurian Sea (43°57'N – 9°41'E)	76	311.64 ± 31.37 (250–410)	216.00 ± 73.87 (106–513)	41	35
WS – West Sardinian Sea (40°28'N – 08°05'E)	34	331.47 ± 83.43 (260–540)	349.91 ± 314.36 (147–1218)	314	106
FR – Gulf of Lion (43°19'N – 4° 6' E)	49	315.93 ± 25.47 (265–380)	237.67 ± 59.70 (139–390)	11	5
BAL – Balearic Sea (40°23'N – 4°28'E)	30	390.66 ± 36.33 (320–450)	522.03 ± 153.01 (291–939)	23	15
AL – Alboran Sea (36°34'N – 3°30'O)	122	309.82 ± 41.13 (255–470)	236.61 ± 111.10 (123–982)	89	70

Pearson), abundance (A), mean intensity (MI), and range of larvae present (min – max), following Bush et al. (1997), Rozsa et al. (2000) and Reiczigel (2003). They were estimated using the Quantitative Parasitology Qpweb software, implemented for the web (Reiczigel and Rozsa, 2005). The statistical significance of the differences observed in the prevalence and abundance values of the infection by *Anisakis* spp. larvae observed was assessed by the Fisher's exact test and Bootstrap *t*-test, respectively (Reiczigel and Rozsa, 2005). Differences were considered significant when $p < 0.05$.

Subsequently, all the samplings data from different areas were pooled, and a Generalized Linear Models (GLMs) were implemented to test for the effect of sampling zone (A) and fish length (L) on the parasitic abundance (n). Abundance data were log-transformed ($\log n + 1$), in order to fit a normal distribution. Two different GLMs were implemented to test for the effect of the explanatory variables and their dependence/independence: a) GLM₁, independence between variables, b) GLM₂, full dependence between variables. Models were compared by using the Akaike's information criterion (AIC, Burnham and Anderson 1998), and results were expressed as AIC differences (ΔAIC), with the best model having $\Delta AIC = 0$.

Correlation between fish length and abundance values of the parasites in the viscera, ventral flesh, and dorsal flesh, were tested by means of a 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, a General Linear Model (GLM), which can accommodate for various non-normal error distributions of data, was run for the samples obtained from different geographic areas. For the parasitic abundance in fish, a logistic regression model with a binomial error distribution and logit link function was applied. Thus, the G statistic (i.e. the chi-squared 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 = 28783), first identified to genus level, were subsequently recognized as larval Type I (28721) and Type II (62) on the basis of available morphological characters (*sensu* Berland, 1961).

According to the alleles observed at the diagnostic loci, i.e., *Adk-2*¹⁰⁰, *Pep C-1*¹⁰⁰ and *Pep C-2*¹⁰⁰ (Mattiucci et al., 2014b), the subsample of 813 *Anisakis* type I larvae from all fish batches were assigned to the species *A. pegreffii*; according to the diagnostic alleles *Adk-2*¹⁰⁵, *Pep C-1*⁹⁰ and *Pep C-2*⁹⁶ (Mattiucci et al., 2014b), one single larva was assigned to *A. simplex* (s. s.). Finally, 62 type II larvae resulted as *A. physeteris* according with diagnostic loci, i.e., *Adk-2*⁹⁷, *Pep C-1*¹¹⁰ and *Pep C-2*¹⁰⁸ as indicated in Mattiucci et al., 2001. One single larva showed a heterozygote pattern between *A. pegreffii* and *A. simplex* (s. s.) at all the three allozyme diagnostic loci.

Anisakis specimens (N = 150) previously identified by those diagnostic allozymes and sequenced at the nuclear elongation factor EF1 α -1 of nDNA, were recognized as belonging to *A. pegreffii* (N = 0.149) or to *A. simplex* (s. s.) (N = 0.1) (Fig. 3). Additionally, the same specimen (N = 1) that showed a heterozygote pattern between alleles of *A. pegreffii* and *A. simplex* (s. s.) at the three allozyme diagnostic loci, had two peaks at both the two diagnostic positions of the EF1 α -1 nDNA locus existing between the two *Anisakis* species (Fig. 3b); thus, two genetic markers agreed in identifying this individual as an F1 hybrid. The nuclear elongation factor EF1 α -1 of nDNA sequences were deposited in GenBank (accession numbers: KY595203, KY595204, KY595205, KY595206, KY595207, KY595208, KY595209, KY595210 for *A. pegreffii*, MF374708 for *A. simplex* (s. s.), and MF358544 for the

F1 hybrid specimen).

Finally, *Anisakis* specimens (N = 240) among those previously identified by allozyme diagnostic loci and EF α -1 of nDNA, sequenced at the mtDNA *cox2* locus, confirmed the identification of *A. pegreffii*, *A. simplex* (s. s.) and *A. physeteris* in accordance with the previous results. The specimens matched, respectively, 100 or 99% with the *A. pegreffii*, *A. simplex* (s. s.) and *A. physeteris* sequences previously deposited in GenBank (Valentini et al., 2006; Mattiucci et al., 2014b). Phylogenetic analysis inferred from BI (Fig. 2) showed that those sequences (N = 69) obtained at the mtDNA *cox2* gene from those *Anisakis* spp. larvae, clustered in a well supported clade also including the sequence of *A. pegreffii*, for that gene, previously obtained and deposited in GenBank. Similarly, the sequence of the single one specimen of *A. simplex* (s. s.) clustered in the clade represented by specimens of *A. simplex* (s. s.) sequenced in previous analyses and deposited in GenBank (Mattiucci et al., 2014b). Finally, *A. physeteris* sequences (N = 9) clustered in the clade formed the previously deposited sequence of that species (Fig. 2).

The F1 hybrid between *A. pegreffii* and *A. simplex* (s. s.) detected by the two nuclear markers, when inferred from mtDNA *cox2* sequence analysis, showed a matrilineage as *A. pegreffii*.

Sequences of mtDNA *cox2* were deposited in GenBank (accession numbers: KY595195, KY595196, KY595197, KY595198, KY595199, KY595200, KY595201, KY595202 for *A. pegreffii*, KY595211, KY595212, KY595213 for *A. physeteris*, and MF358545 for *A. simplex* (s. s.)).

Besides larval specimens of *Anisakis* spp., 16 specimens of the raphidascaridid nematodes belonging to the genus *Hysterothylacium* spp., all detected in the visceral cavity of the fish host, were identified to the species level as *H. aduncum*. The sequences obtained (936 bp) matched at 99% with the ITS rDNA sequence of *Hysterothylacium aduncum* deposited in GenBank (JQ934878) (Smrzlić et al., 2012). Sequences of ITS rDNA for *H. aduncum* were deposited in GenBank under the accession numbers: KY595214, KY595215.

3.2. Distribution of *A. pegreffii* by location, fish size and fishing ground

A. pegreffii larvae were detected in the viscera, in the ventral, and in the dorsal flesh of the examined fish. They were recovered in syntopy with larval *A. physeteris* in the viscera of some of the hakes examined. Indeed, *A. physeteris* larvae were never found infecting the fish fillets. The single specimen belonging to the species *A. simplex* (s. s.) was recovered in syntopy with *A. pegreffii* in the viscera of the same individual fish host caught from the Alboran Sea. The F1 hybrid specimen so far identified was detected in this same batch of hake.

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

No statistically significant differences were observed in terms of prevalence and mean abundance of the infection with *A. pegreffii* between batches of hakes sampled from the same locality in different seasons of the year (p always > 0.05). As a consequence, the data were pooled and identified only by the fishing grounds.

The overall levels of infection by *A. pegreffii* of hake samples originating from different Mediterranean fishing grounds showed different values ($p < 0.001$) (Table 2). In addition, fish from each area resulted infected, even when showing different mean parasite abundances. Indeed, *A. pegreffii* exhibited the highest levels of infection both in prevalence (P = 100.0) and mean abundance (A = 157.3, range 4–866) (Table 2) in hakes sampled in the southwest of the Adriatic Sea (SA – sampling area 4 in Fig. 1, and Table 2). High levels of infection (prevalence P = 89.8; mean abundance A = 98.9) were recorded also in hakes sampled in the central western area of the Adriatic Sea (CA in Table 2), despite the average smaller size of the fish obtained from this area (mean fish length = 311.5 cm). Remarkably high values of infection (prevalence P = 91.8; mean abundance A = 15.88) have also been

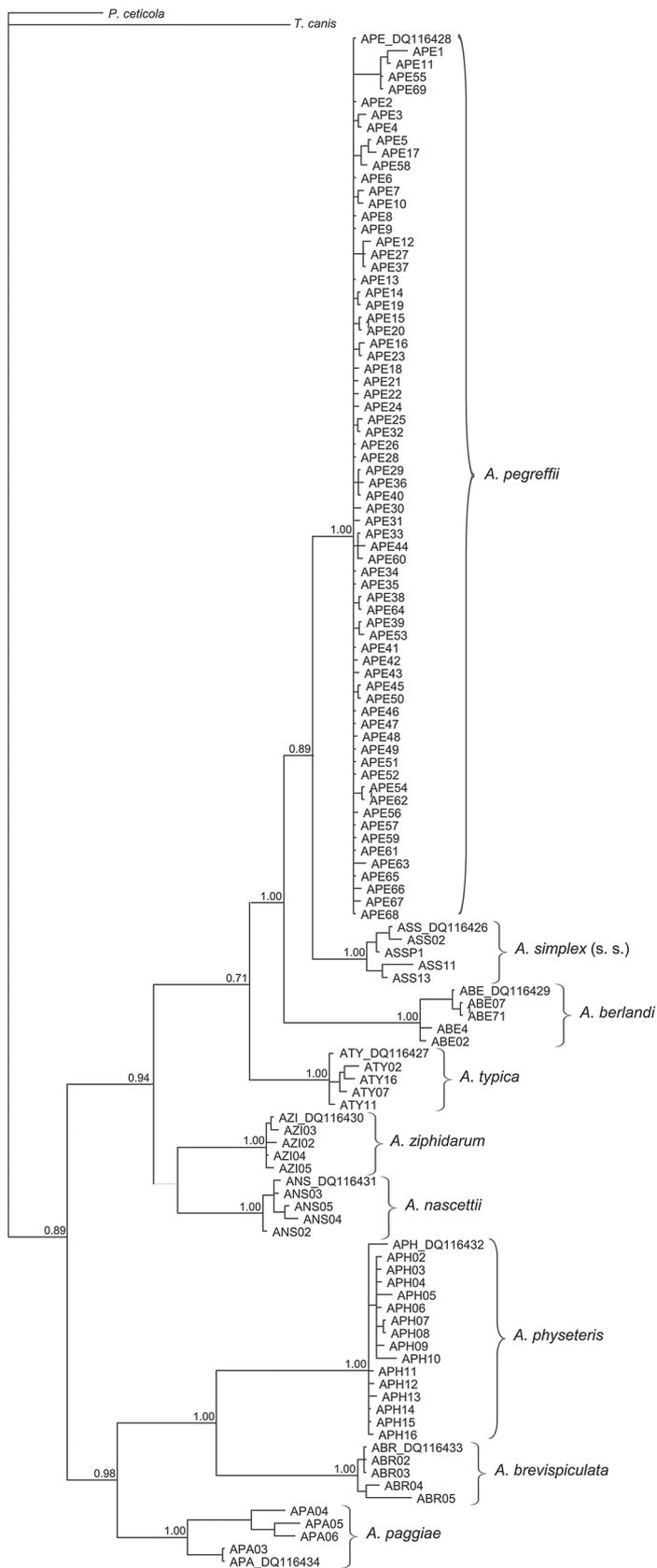


Fig. 2. Bayesian inference (BI) tree based on mtDNA *cox2* gene sequences of *A. pegreffii* larvae from *M. merluccius* sampled in the Mediterranean Sea, with respect to the other *Anisakis* spp. previously sequenced. The analysis was performed by MrBayes3.1 (Huelsenbeck and Ronquist, 2005), using TIM1 + I + G substitution model, as implemented in jModeltest2.1 (Darriba et al., 2012); the parameters for selected model are I = 0.392 and G = 0.463, calculated with Akaike Information Criterion (AIC) (Posada and Buckley, 2004). For the Bayesian analysis, four incrementally heated Markov Chains (using default heating values), were run for 1,000,000 generations, sampling the Markov Chains at intervals of 100 generations. Numbers at the nodes are posterior probabilities. *T. canis* and *P. ceticola* were used as outgroups.

recorded in the central eastern Adriatic Sea (CR), in the northern area of the same basin (NA in Table 2, P = 87.5; A = 9.29), and in the Ionian Sea area, off Patras' coast (IO in Table 2, showing P = 55.8 and A = 2.62 values). Focusing on hake samples from different areas of the

Adriatic Sea basin, we observed congruence across specific areas. Likewise, hakes from central Adriatic off the Italian coast (CA) and off the Croatian coast of Split (CR) showed similar prevalence (P = 89.4 and P = 91.8, respectively; $p = 0.54$), but different mean abundance

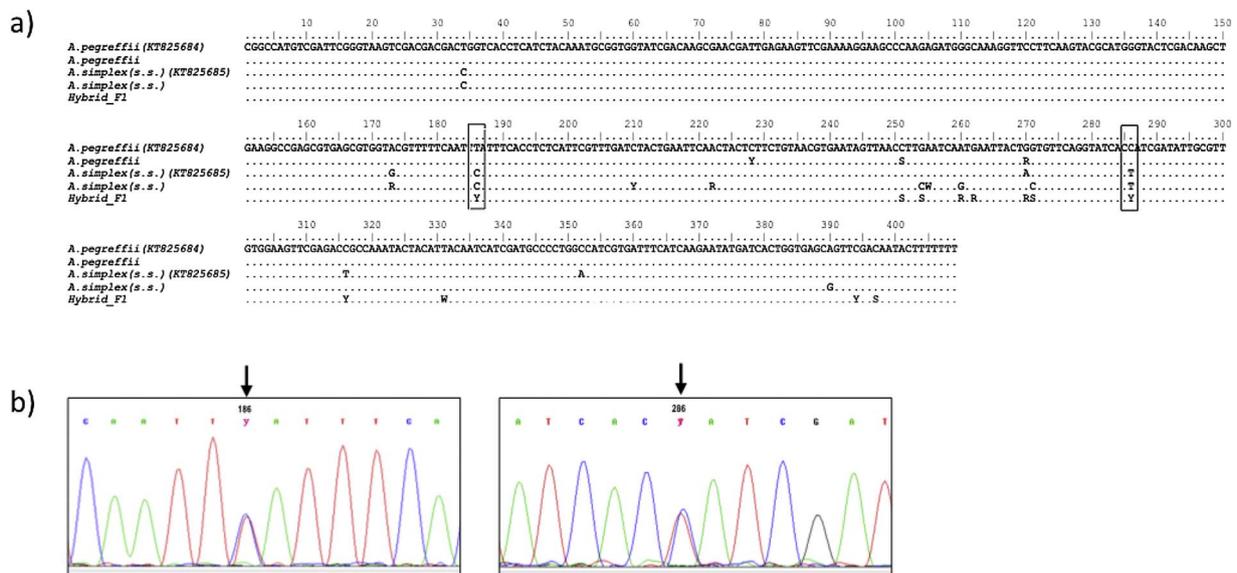


Fig. 3. a) Alignment of the elongation factor (EF1 α -1 nDNA) nuclear gene in the two species *A. pegreffii* and *A. simplex* (s. s.) and of the F1 hybrid specimen found between the two species. Dots indicate identity; standard IUPAC codes were used, i.e., Y = C/T. The rectangles underlined the two diagnostic nucleotide positions between *A. pegreffii* and *A. simplex* (s. s.). b) The heterozygote patterns of the F1 hybrid between the two species are highlighted by the arrows at the two diagnostic nucleotide positions (bp 186 and 286).

levels ($A = 32.20$ and $A = 13.72$, respectively; $p = 0.001$), whereas the mean length of the hakes differed (311.45 ± 74.44 cm and 212.00 ± 62.46 cm, respectively $p < 0.01$) (Table 1).

Overall, the hake samples from the other fishing grounds of the western Mediterranean Sea showed lower levels of infection compared to the fish originating from the Adriatic/Ionian area, with statistically significant differences in term of prevalence and abundance of infection ($p < 0.05$) (Table 2).

Concerning the western sampling sites of the Mediterranean Sea, higher levels of infection, in term of prevalence and abundance of infection, have been recorded in hakes sampled in the waters off the South of Sicily (SS in Table 2, $P = 57.8$; $A = 6.4$) and in the North West of Sardinian Sea (WS in Table 2, $P = 55.9$; $A = 9.1$). The samples from the Alboran Sea, Balearic Sea, Ligurian Sea and Gulf of Lion showed the lowest levels of infection, including both prevalence and abundance (Table 2).

The distribution of *A. pegreffii* larvae by site of the infection in the host, such as liver, viscera, ventral flesh and dorsal flesh, was reported as relative frequency of larvae counted per site. No statistically significant differences after a Mann-Whitney test ($p > 0.05$) was found among the relative frequencies observed in each hake sample from different localities. Thus, the relative proportions of *A. pegreffii* by infection sites in the fish host was cumulated for the different batches, and resulted as following: 28.3% of *A. pegreffii* larvae were detected in the fish liver, whereas 62.9% in the rest of the host's viscera; 6.6% in the ventral part of the fish flesh and 2.1% in the dorsal flesh of infected hakes.

Taking into account the whole sample of hakes, the best model explaining the parasitic burden by *A. pegreffii* in *M. merluccius* was the GLM₂. This model yielded a correlation between the abundance of parasites and both the fish length (L) and the sampling area (A). Indeed, differences in length have been found between hakes from different fishing grounds of the Mediterranean Sea (Student's *t*-test, $t = 15.32$, $p < 0.001$). A significant correlation ($r^2 = 0.349$, $p < 0.001$) between fish length (L) and number of parasites (n) resulted by a Spearman correlation analysis, with the total (flesh and viscera) number of larvae increasing with the length of the fish host (Fig. 4). A significant correlation was also recorded between fish length and the number of larvae detected in the ventral flesh ($r^2 = 0.284$, $p < 0.001$), and between fish length and the number of larvae detected in the dorsal flesh ($r^2 = 0.193$, $p < 0.001$). Moreover, a significant correlation was

recorded between the value of the parasitic abundance observed in the ventral flesh with those detected in the dorsal flesh ($r^2 = 0.322$, $p < 0.01$), and finally between the abundance value recorded in the viscera of the fish with respect of those estimated in the fish flesh ($r^2 = 0.345$, $p < 0.01$).

According to the results obtained by the GLM₃ model, the main variables associated with the *A. pegreffii* parasite burden in hake samples were fish length and fishing ground. Fulton K parameter, related to the body condition index (BCI) of the fish, did not show any significant correlation when related with the abundance of *A. pegreffii* infection.

3.3. Distribution of *A. physeteris* and *H. aduncum* larvae by site of infection, fish length and fishing ground

A total of 62 *Anisakis* type II larvae identified by genetic/molecular markers as belonging to *A. physeteris* species were found in hakes sampled from some of the fishing grounds. The highest levels of infection by *A. physeteris* was recorded in the Alboran Sea, where a prevalence $P = 13.9$ and abundance $A = 0.35$ values of the infection by that parasite species were recorded. A small number of *A. physeteris* larvae (< 10 specimens per area) were also detected in fish sampled at WS, IO, CT.

Hysterothylacium spp. larvae were sporadically detected ($N = 17$) only in *M. merluccius* sampled in the Adriatic Sea area (CR and SA), showing low levels of infection. All the larvae of *Hysterothylacium* spp. detected were found in the visceral cavity of the fish host.

4. Discussion

Larval specimens belonging to three species of *Anisakis* (i.e. *A. pegreffii*, *A. physeteris*, and *A. simplex* (s. s.)) were identified in a large sample of European hakes obtained from 13 different fishing grounds of the Mediterranean Sea. *A. pegreffii* was detected in fish collected from all fishing grounds; it also occurred in sympatry and syntopy with *A. physeteris* from hakes fished in four sampling areas (AL, SA, IO, CT) (Fig. 1). The only *A. simplex* (s. s.) larva identified was collected in a fish from the Alboran Sea (AL), in syntopy with other *A. pegreffii* larvae. *A. simplex* (s. s.) was reported with higher frequencies in this area of the Mediterranean Sea always in sympatry with *A. pegreffii* in previous studies (Mattiucci et al., 2004), but also in parasitological surveys of other fish species, such as *Lepidopus caudatus* and *Lophius piscatorius*,

Table 2

Prevalence (P, %) with confidence limits (Clopper-Pearson), mean abundance (A), and range (min – max) of *Anisakis pegreffii* larvae detected in *Merluccius merluccius* samples from each sampling areas. Number of total larvae (N_{TOT}) and their relative proportions (%) in different tissues are also given.

		NA	CA	CR	SA	IO	EG
Viscera	N FISH	73	137	257	46	50	27
	P%	84.7	89.8	89.9	100.0	90.0	74.1
		0.743–0.921	0.833–0.939	0.855–0.930	0.932–1.000	0.782–0.367	0.430–0.798
	A (RANGE)	7.86 ± 7.19 (2–31)	96.09 ± 362.41 (1–3005)	14.34 ± 40.14 (1–383)	145.5 ± 167.73 (3–773)	40.14 ± 109.30 (1–602)	2.22 ± 2.14 (1–7)
Tot (%)	566 (84.6%)	13165 (97.1%)	3685 (90.3%)	6697 (92.5%)	2007 (98.1%)	60 (87.0%)	
Ventral flesh	P%	48.6	51.8	19.8	87.0	28.8	19.2
		0.36–0.60	0.434–0.602	0.152–0.252	0.737–0.951	0.162–0.425	0.102–0.415
	A (RANGE)	0.86 ± 7.86 (1–5)	2.35 ± 4.25 (1–29)	0.33 ± 0.83 (1–17)	8.63 ± 13.74 (1–62)	0.66 ± 1.35 (1–7)	0.23 ± 0.51 (1–2)
	Tot (%)	71 (10.6%)	322 (2.4%)	86 (2.1%)	397 (5.5%)	33 (1.7%)	7 (10.1%)
Dorsal flesh	P%	22.2	33.6	40.1	65.2	8.0	7.4
		0.13–0.22	0.258–0.419	0.342–0.463	0.498–0.786	0.022–0.192	0.13–0.237
	A (RANGE)	0.44 ± 1.02 (1–5)	0.55 ± 1.01 (1–6)	1.206 ± 2.615 (1–6)	3.11 ± 6.25 (1–31)	0.100 ± 0.30 (1–2)	0.07 ± 0.27 (1–1)
	Tot (%)	32 (4.8%)	75 (0.5%)	310 (7.6%)	143 (2.0%)	5 (0.2%)	2 (2.9%)
Total	P%	87.5	89.8	91.8	100.0	94.0	81.5
		0.77–0.94	0.837–0.940	0.879–0.947	0.932–1.000	0.835–0.987	0.538–0.876
	A (RANGE)	9.29 ± 8.05 (1–34)	98.9 ± 366.13 (2–3037)	15.88 ± 41.80 (1–399)	157.32 ± 182.56 (4–866)	40.90 ± 110.18 (1–608)	2.55 ± 2.25 (1–8)
	TOT	669	13562	4081	7237	2045	69

		SS	CT	LI	WS	FR	BAL	AL
Viscera	64	165	76	34	49	30	122	
	51.6	40.6	25.0	55.9	14.3	41.9	11.8	
	0.390–0.639	0.330–0.485	0.158–0.363	0.381–0.724	0.059–0.272	0.245–0.609	0.191–0.347	
	5.84 ± 15.51 (1–98)	1.28 ± 2.50 (1–17)	0.50 ± 1.27 (1–7)	8.58 ± 24.09 (1–121)	0.16 ± 0.43 (1–2)	0.74 ± 1.26 (1–6)	0.62 ± 1.27 (1–3)	
	374 (90.1%)	211(91.7%)	38 (92.7%)	292 (92.9%)	8 (72.7%)	23(100.0%)	76 (85.4%)	
Ventral flesh	26.6	6.1	2.6	23.5	6.1	0.0	7.4	
	0.170–0.390	0.029–0.109	0.003–0.092	0.114–0.411	0.013–0.169	0.000–0.000	0.037–0.133	
	0.14 ± 0.69 (1–9)	0.08 ± 0.37 (1–3)	0.02 ± 0.16 (1–1)	0.44 ± 0.92 (1–4)	0.06 ± 0.24 (0–1)	0.00 ± 0.00 (0–0)	0.10 ± 0.37 (1–2)	
	29 (7.0%)	14 (6.1%)	2 (4.9%)	19 (6.1%)	3 (27.3%)	0	12 (13.5%)	
	Dorsal flesh	6.30	3.0	1.3	8.8	0.0	0.0	0.8
0.022–0.154		0.010–0.069	0.000–0.071	0.024–0.232	0.000–0.073	0.000–0.112	0.000–0.042	
0.14 ± 0.69 (1–5)		0.03 ± 0.17 (1–1)	0.01 ± 0.11 (1–1)	0.08 ± 0.28 (1–3)	0.00 ± 0.00 (0–0)	0.00 ± 0.00 (0–0)	0.01 ± 0.09 (1–1)	
9 (2.9%)		5 (2.2%)	1 (2.4%)	3 (1.0%)	0	0	1 (1.1%)	
Total		57.8	44.2	27.6	55.9	20.4	41.9	26.2
	0.453–0.697	0.365–0.525	0.180–0.391	0.381–0.724	0.102–0.343	0.245–0.609	0.203–0.364	
	6.43 ± 16.50 (1–98)	1.39 ± 2.56 (1–17)	0.54 ± 1.29 (1–7)	9.11 ± 24.81 (1–124)	0.22 ± 0.47 (1–2)	0.74 ± 1.26 (1–6)	0.73 ± 1.47 (1–3)	
	412	230	41	314	11	23	89	

run in the framework of the PARASITE Project (introduction paper of this special issue, [Levsen et al., in press](#)). In the same sample from the Alboran Sea, also a F1 hybrid between *A. pegreffii* and *A. simplex* (s. s.) was found, having a matrilineage as *A. pegreffii* (inferred from mtDNA *cox2* sequence analysis, data not shown). The detected F1 individual is the result of a current hybridization event in a sympatric area of the two species in the Mediterranean Sea. On the other hand, Alboran Sea represents an oceanographic transition zone between Atlantic Ocean and Mediterranean Sea ([Tintore et al., 1988](#)). From this water basin, other *Anisakis* spp. larvae have been recorded in other fish species ([Mattiucci et al., 2014a](#); [Levsen et al., in press](#)).

Anisakis pegreffii infection exhibited significantly higher values of infection than *A. physeteris* in hakes from all sampled fishing grounds. The highest values of prevalence (P = 13.9) and abundance (A = 0.35) for *A. physeteris* were recorded in the Alboran Sea. These values were lower than those detected for *A. pegreffii* (P = 26.2; A = 0.73) infection

from the same fishing ground. In all other sampling areas, *A. pegreffii* larvae outnumbered *A. physeteris*. The overall higher levels of infection by *A. pegreffii*, with respect to *A. physeteris*, are generally similar to those previously reported for these two species in European hake from the same fishing grounds ([Table 3](#)) ([Mattiucci et al., 2004](#)), even though the levels of infection in the flesh of this fish species were previously not reported, thus slightly underestimating the quantification of *A. pegreffii* in the Mediterranean hakes. Furthermore, *A. pegreffii* larvae occurred in the viscera and flesh of the fish, whereas all the larvae of *A. physeteris* were found only in the viscera (mostly embedded in the liver) of the host. It can be therefore hypothesized that the latter species is not capable of migrating in the musculature of the fish.

The parasitic levels of infection by *A. pegreffii* recorded in our *M. merluccius* samples from different fishing grounds throughout the Mediterranean Sea were extremely heterogeneous, and in agreement with the scarce literature data present so far ([Table 3](#)). This different

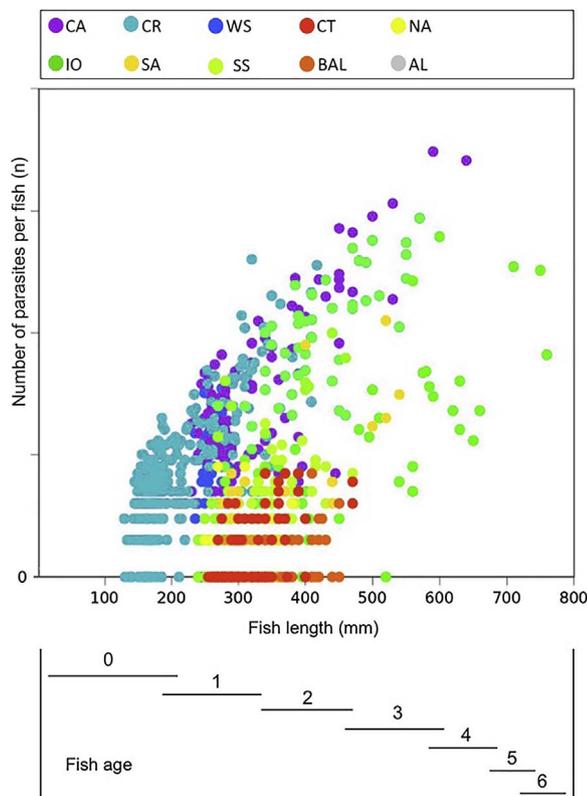


Fig. 4. Spearman correlation analysis ($r^2 = 0.349$, $p < 0.001$) between fish total length (mm) and number of parasites per fish (n). Below, estimate of the fish age based on length, according to Mellon-Duval et al., 2010. Colour spots are associated to the different sampling localities, as indicated above.

parasitic burden recorded in different fish samples seemed to be addressed by two drivers of the infection: the fish size and the fishing ground. The best model explaining the parasitic burden by *A. pegreffii* recorded in hakes was the GLM₂, which highlighted the correlation between the abundance of parasites with both the fish length (L) and the sampling area (A). In our samples, the fish length was the main variable related to the abundance of this parasite species.

Likewise, hakes from the western and eastern part of the central Adriatic Sea showed similar prevalence ($P = 89.4$ and $P = 91.8$; $p = 0.54$) but different mean abundance values ($A = 32.20$ and $A = 13.72$, $p = 0.001$ respectively in hakes from Italian coasts versus Croatian ones). Thus, this difference in abundance of larvae could be explained by the highest length recorded for the fish obtained off the Italian coast compared to the batch originating from the Croatian coast (311.45 ± 74.44 cm and 212.00 ± 62.46 cm, $p < 0.01$). In all *M. merluccius* samples examined, larger fish tended to show (flesh plus viscera) a higher abundance of the parasite (Spearman correlation $r^2 = 0.349$, $p < 0.001$, Fig. 2) (length/age relation was estimated by Mellon-Duval et al., 2010, fitting a von Bertalanffy model to data obtained from recaptured fish, in the Gulf of Lion). Considering the whole examined sample of *M. merluccius* and analyzing it with a higher *A. pegreffii* burden, it is evident that these specimens corresponded to the 4th–7th year of age (range: 60 cm–80 cm of length) (Fig. 2) suggesting a direct relation with feeding habits of the fish, preying upon zooplanktonic organisms and other fish species that act as hosts of *A. pegreffii* larvae. Considering that once *A. pegreffii* larvae accumulate in the fish host, where they probably remain an undetermined time, the correlation between the size of the fish and the parasitic burden is a direct consequence of the time that the fish spent feeding on its prey. Hake is a top predator that occupies different trophic levels during its life, and its diet shifts from euphausiids and decapods, consumed by the smaller individuals (< 16 cm TL), to fishes consumed by larger

individuals (Carpentieri et al., 2005). Before the full transition to the ichthyophagous phase, hake showed more generalized feeding habits where decapods, benthic (Gobiidae, *Callionymus* spp., *Arnoglossus* spp.) and nektonic fish (*Sardina pilchardus*, *Engraulis encrasicolus*) are predominant in the diet, whereas cephalopods have a lower incidence (Carpentieri et al., 2005).

In some larger hakes fished in central Adriatic Sea (CA), the highest amount of larvae per single infected fish was recorded: a 59 cm long specimen harboured 3037 larvae, of which 3005 were located in the viscera and 22 in the muscular part. Also in the Ionian Sea off Patras (IO), some fish of large size showed high levels of infection; 608 larvae were counted in a single hake, whose length was 60 cm (Fig. 2).

The geographical areas too, with their ecological conditions and the presence of suitable hosts for the life cycle of the *Anisakis* spp., seem to represent a determinant driver of *A. pegreffii* rate of infection. Previous authors (Valero et al., 2006; Mattiucci et al., 2004, 2014b; Suzuki et al., 2010; Cipriani et al., 2017) noted that levels of infection by different species of *Anisakis* vary depending on the geographical origin of the fish host. In fact, remarkably higher levels of infection for *A. pegreffii* were recorded in hakes of the Adriatic Sea/Ionian Sea areas compared to the fish of the same size range obtained from the western Mediterranean fishing grounds. The highest levels of infection (both in prevalence $P = 100.0$ and mean abundance $A = 157.3$, range 4–866; Table 2) were recorded in hakes fished off the Italian coasts of the southern area of the Adriatic Sea (SA – sampling area 4 in Fig. 1, and Tables 1 and 2). Notably, the sample of fish from this area was larger (mean length 415.97 cm) (SA in Table 1) compared with the other fish batches of the Adriatic Sea (CA and CR in Table 1) ($p < 0.001$). High levels of infection have been also recorded in hakes caught off the Italian coasts of the central area of the Adriatic Sea (prevalence $P = 89.8$ and mean abundance $A = 98.9$), despite of the overall smaller size of fish sampled in this area (mean length 311.5 cm, CA in Table 1).

Concerning the western Mediterranean fishing ground, the hakes sampled from the central Tyrrhenian Sea (CT) showed low levels of infection of *A. pegreffii* (Table 2), with values comparable to those obtained in samples from Aegean Sea (AEG), Ligurian Sea (LI) and Balearic Sea (BAL) ($p > 0.05$) (Table 2).

The eastern basin of the Mediterranean Sea, in particular the central and southern area of the Adriatic Sea, appears as a “hotspot” for the presence of *A. pegreffii*. This statement is supported by data recorded for other fish species, like *Engraulis engraulis* (Cipriani et al., 2016, 2017) and *Sardina pilchardus* (Levsen et al., in press), showing the highest levels of infection in this western part of the Mediterranean Sea (Table 2). This finding could be related to ecological characteristics of the Adriatic Sea, with both abiotic and biotic conditions favourable to maintain the life cycle of *A. pegreffii* at high population size (see Cipriani et al., 2017). Some favourable abiotic conditions of the central Adriatic Sea, which favour the presence of a stable food web, may result in enhancing the overlapped and abundant distribution of hosts belonging to different trophic levels of the life cycle of *A. pegreffii*, and could thus explain its pattern of distribution and high levels of infection in different fish species, such as hakes, anchovies (present issue Cipriani et al., 2017), and sardines (Levsen et al., in press), in this semi-enclosed and narrow basin. Abiotic and biotic factors, such as mean sea surface temperature, depth, salinity, sea surface temperature range, as well as primary production, were identified as most important abiotic variables impacting the distribution of *Anisakis* spp. (Højgaard, 1998; Kuhn et al., 2016).

A further hypothesis to explain the high densities of *A. pegreffii* recorded in this basin could be the presence of an “anthropogenic shortcut” in the life cycle of the parasite (see Cipriani et al., 2017). There is a widely used routine, followed by local fishermen, who eviscerate fish (above all hakes and scabbard fish) directly on board of the vessels, then discard the viscera at sea, together with bycatches. This practice seems related to the necessary removal of viscera in fish species largely infected by *Anisakis* larvae, whose presence would

Table 3

Historical data from recent scientific literature on *A. pegreffii* and *A. physeteris* infection levels recorded in *M. merluccius* from the localities of the Mediterranean Sea here studied. Year of sampling, number of fish examined (N_{fish}) with fish mean length (n. r. stands for not reported), Prevalence (P) and Abundance (A) are reported.

Fishing area	Year of sampling	N_{fish} mean length (mm)	<i>A. pegreffii</i>		<i>A. physeteris</i>		Author
			P	A	P	A	
Croatian coast	2014	120 (342.92 mm)	70.8	6.87	–	–	Mladineo and Poljak (2014)
West Sardinian Sea	2014	96 (200.00 mm)	37.5	–	1.0	–	Piras et al. (2014)
Sardinian Sea	2011	50 (n. r.)	30.0	0.5	–	–	Angelucci et al. (2011)
Sardinian Sea	2011	50 (n. r.)	–	0.32	–	–	Meloni et al. (2011)
Alboran Sea	1994	63 (450.00 mm)	0.71	–	0.01	–	Valero et al. (2006)
Aegean Sea	2012	10 (262.00 mm)	80.0	9.6	–	–	Pekmezci et al. (2014)
Ionian Sea	2004	53 (n. r.)	94.2	30.4	3.3	0.1	Mattiucci et al. (2004)
Adriatic Sea	2004	60 (n. r.)	73.3	10.0	–	–	Mattiucci et al. (2004)
Tyrrhenian Sea	2004	67 (n. r.)	35.8	0.8	6.0	0.1	Mattiucci et al. (2004)
Ligurian Sea	2004	84 (n. r.)	63.8	71.7	3.6	0.1	Mattiucci et al. (2004)
Balearic Sea	2004	77 (n. r.)	8.3	0.1	31.6	0.6	Mattiucci et al. (2004)
Aegean Sea	2004	90 (n. r.)	54.4	12.1	1.1	0.1	Mattiucci et al. (2004)

compromise the value of the fish at landing. At the beginning of this study, we had several problems in obtaining “intact” hake specimens, because most fishermen had the habit of removing part of the viscera of hake straight onboard of trawlers, ripping away gills and linked organs straight from the gills holes, without cutting the fish directly. These viscera discards, originating from various commercial fish species of that area, would have been dropped at sea together with the common bycatch discards, including still alive *Anisakis* larvae. Once at sea, these discards may represent a food source for fish, cetaceans and seabirds, that may eventually acquire *Anisakis* larvae present; it is in fact suggested that discards from fishing may increase the parasite transmission to the next host (Oro and Ruiz, 1997; Morton and Yuen, 2000; Arcos et al., 2001; Bozzano and Sardà, 2002). In Northern areas of the Adriatic Sea dolphins distribution overlaps with areas used intensively by fishermen and many sightings were recorded during interactions with fishery (Genov et al., 2008), when dolphins have been observed feeding on discarded fish as well (Bearzi et al., 2008). Animals follow working nets (feeding on stirred-up organisms or fish gilled in mesh) and also feed on discarded by-catch (Fertl and Leatherwood, 1997). We therefore hypothesize that fish and dolphins feeding on these by-catches discarded at sea, belonging to the same heavily parasitized fish of the batch we inspected, could acquire greater amount of *Anisakis* individuals, enhancing this “anthropogenic shortcut” in the parasite cycle.

According to the GLMs models, no statistically significant correlation was found between the Fulton K BCI of the hakes and the *A. pegreffii* abundance ($p > 0.05$). Both fish from heavily infected stocks and even fish that resulted not infected, maintained a constant proportion in weight and length, showing no change in their body condition index (BCI), and thus likely suggesting that these larval parasites have no pathogenic impact on the fish health status.

Since *M. merluccius* is one of the most important European fish species caught off western Europe, this study offers a crucial food safety device for assessing risks associated with these parasites in hakes from several fishing areas of the Mediterranean Sea. The fillets, as the edible part of the fish, represent the real risk to the consumer when harbouring zoonotic *Anisakis* larvae. Even if there is no evidence of large consumption of raw hake meat in the European Mediterranean countries, as it happens for anchovies or sardines, the exact information on larval localization in the fish product should be provided for a detailed risk assessment to the consumer (Bao et al., 2017). At least, not only live larvae could cause the parasitic zoonosis (human anisakiasis), but even dead larvae could provoke allergic symptoms in sensitized patients (Audicana and Kennedy, 2008). Therefore, an accurate examination of the fish flesh permits a real assessment of the parasitic levels of infection to better evaluate the real threat of human infection. A significant correlation was recorded between the abundance of *A. pegreffii* larvae

present in the viscera of the hakes and the larvae counted in the flesh ($r^2 = 0.345$, $p < 0.01$). This result is consistent with similar observations reported by Karl and Levsen (2011) in grey gurnards infected by *A. simplex* (s. s.). Indeed, these authors reported in this fish “...a significantly positive relationship between both abundance and intensity of the larvae (*A. simplex* (s. s.)) in the flesh and the viscera ($r = 0.64$ and $r = 0.59$, respectively), i.e. the number of larvae in the flesh tend to increase with increasing infection level in the viscera...”.

Only 2.5% of the fish positive for *A. pegreffii* exhibited an infection in the flesh and no larvae in the viscera, with a maximum number of three larvae detected in the ventral part of the fillet. Karl and Levsen, 2011 reported a similar observation on a different *Anisakis* species infecting a different fish host, with the 1.8% of *A. simplex* (s. s.) larvae being in flesh of grey gurnard, but not in viscera. Those authors emphasized that fish which “appear to be free of nematodes in the viscera may still carry *A. simplex* larvae in the flesh, i.e. the larval infection level in the viscera cannot be used as reliable indicator for the approximate *Anisakis* burden in the flesh (Karl and Levsen, 2011)”.

Finally, the correct genetic/molecular identification of the anisakid nematodes involved in the fish infection represents the basis for any epidemiological survey intended to identify the zoonotic species involved and enhances the possibility to provide an accurate human risk assessment. Different *Anisakis* species showed different capacities of migrating in the muscular edible part of the fish, aspect that involves a different zoonotic potential. For example, *A. physeteris* was never reported in the fish flesh. In addition, no human cases of anisakiasis have been so far recorded as caused by this species. Data so far acquired suggest that *A. pegreffii* and *A. simplex* (s. s.) differ in their site of infection in the fish host, with *A. simplex* (s. s.) showing a higher propensity to invade the flesh respect to *A. pegreffii* (Quiazon et al., 2011; Cipriani et al., 2015), even if both species resulted to be zoonotic (D’Amelio et al., 1999; Umehara et al., 2007; Fumarola et al., 2009; Mattiucci et al., 2011, 2013, 2017b; Arai et al., 2014; Lim et al., 2015; Mladineo et al., 2016).

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