Title:

Molecular epidemiology of *Anisakis* spp. in blue whiting *Micromesistius poutassou* in eastern waters of Spain, western Mediterranean Sea*

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Abstract

The infection of blue whiting *Micromesistius poutassou* from the western Mediterranean Sea, off the eastern coast of Spain, with larvae of *Anisakis* spp. was studied. Between April 2016 and April 2017, 140 fish were analyzed. Total epidemiological data showed that the prevalence of *Anisakis* spp. was 29.3% and the mean intensity 1.8. Of the 74 larvae collected, 61% were type I and the remaining 39%, type II. Of the former, 91.1% were molecularly identified as *Anisakis pegreffii* (P=19%; MI=1.4), 2.2% as *Anisakis simplex s.s.* (P=0.7%; MI=1.0), while the rest (6.7%) showed a recombinant genotype between the two (P=2.1%; MI=1.0). All the type II larvae analyzed were molecularly identified as *Anisakis physeteris* (P=10.0%; MI=2.1). Three fish (2.1%) were found to have larvae in the muscle, while two were found with 1 larva of *A. pegreffii* and one with two larvae (1 *A. simplex s.s.* and 1 *A. pegreffii*). Statistical analysis showed that the prevalence of *Anisakis* spp. in blue whiting was higher in spring than in autumn ($p<0.001$), probably due to the greater size (and age) of the fish and related to factors as diet shift, accumulation with age and higher food intake. Analysis of the data suggested that blue

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*Dedicated to Professor Adela Valero, from our Department, on the occasion of her retirement.

whiting were first infected with *Anisakis* type I (mean age 2.3 years) and later with *Anisakis* type II (mean age 2.7 years), probably due to the diet changing with age, with the incorporation of the paratenic/intermediate host species of these parasites. In any case, the public health authorities must continue to emphasize the need for suitable thermal treatment (freezing or cooking) of the fish prior to consumption.

Key words: Anisakiasis; *Anisakis* infection/fish age; *Anisakis pegreffii*; *Anisakis physeteris*.

1. Introduction

The blue whiting *Micromesistius poutassou* is an inexpensive fish which is widely consumed. It is of commercial interest in both the North Atlantic Ocean and the western Mediterranean Sea (WM). The southern blue whiting *Micromesistius australis* is also appreciated in the countries of the southern hemisphere. In 2014, 1.215.616 tons of the two species were landed, ≥ 96% of which was from the Atlantic Ocean, mainly caught by European fishing fleets (Anonymous, 2016), which shows its commercial importance.

Anisakiasis or anisakiosis is the term for human infection by larval nematodes of the genus *Anisakis*. It is most frequently caused by the third-stage larvae (L3) of *Anisakis* type I (*A. simplex s.l.*) (Arizono et al., 2012; Rello Yubero et al., 2004; Romero et al., 2013), generally ingested via the consumption of raw or insufficiently cooked fish. In addition, a few cases of anisakiasis caused by larvae type II of *Anisakis* have been reported (Asato et al., 1991, and references therein; Clavel et al., 1993; Kagei et al., 1978). Recently, Romero et al. (2014), working with rats, have shown that both *A. physeteris* and *A. paggiae*, both larvae type II, are pathogenic, although less so than *Anisakis* type I.

Although the blue whiting is more abundant in the Atlantic Ocean it is also captured in the Mediterranean Sea, for which there have been some studies on infection by *Anisakis* spp. and even fewer where molecular identification of the *Anisakis* larvae has been carried out (Table 1). For this reason, this molecular study of the infection of blue whiting from the western Mediterranean Sea, off the eastern coast of Spain, was carried out, while also analyzing the factors which may affect the infection of these fish with *Anisakis* spp.

2. Materials and methods

2.1. Host and parasites

A total of 140 blue whiting *Micromesistius poutassou* Risso were collected through bottom trawl fishing (depth 40-200 ftm; 38.0-38.5° N and 0.0-0.5° W). The trawlers leave the port at dawn and return 10-12 hours later. The fish were randomly sampled from those landed, immediately after the arrival

of the fishing boats, in the port of Villajoyosa (38.5° N, 0.2° W) in the western WM (Fig. 1), from April 2016 to April 2017 (1 sample in April 2016; 5 in autumn 2016; and 3 in April 2017). The fish were immediately packed in flake ice and transported to the laboratory,, where they were promptly measured, weighed and dissected within 15-18 h of being landed. The "condition factor" of the fish (CF) was calculated using the formula $CF=100\times W/L^3$, where W=total weight (g) and L=total length (cm). This CF is considered as an indicator of general fish health and, according to Monstad (1990), expresses how well nourished the fish are. Following dissection, the larvae of *Anisakis* spp. visible in the visceral cavity were collected. The viscera and muscle of each fish were individually and separately subjected to pepsin digestion at pH 2 and 37 ºC, as described previously (Molina-Fernández et al., 2015), to determine whether larvae were present. Once identified morphologically by optical microscopy as *Anisakis* type I or II (*sensu* Berland, 1961), the larvae were frozen at -20ºC until their preparation for molecular identification. In addition, the age of the fish was determined using the formula $L= 34.26 \cdot (1 - e^{-0.21\ (A+2.58)})$, where L=total length (cm) and A=age (years), after García et al. (1987)

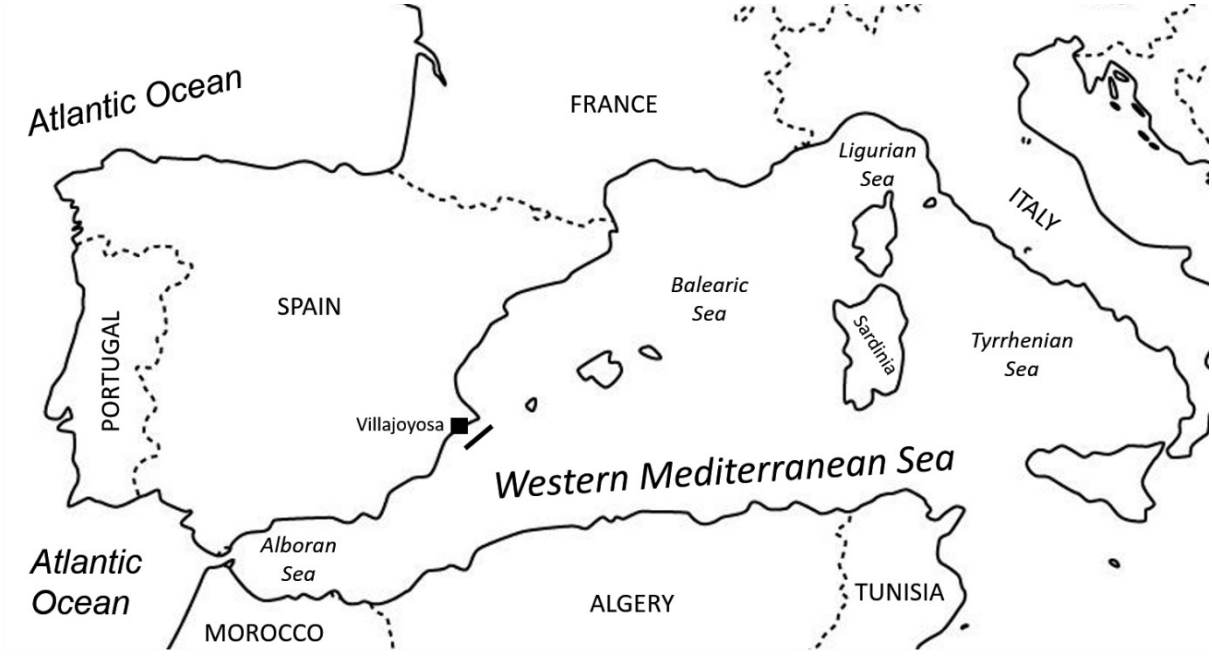

Figure 1.- Geographical area of the western Mediterranean Sea, port (square) where blue whiting were landed and fishing ground (line in front to landing port).

2.2. Molecular identification

Each larva was individually prepared for DNA isolation using the RealPure commercial kit according to the maker's instructions. The fragment of rDNA corresponding to the sequence ITS1-5.8-ITS2 was amplified using the primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (1998). The PCR conditions were as previously described (Molina-Fernández et al., 2015). The expected size of the amplified fragment was around 1000 bp. Next, Restriction Fragment Length Polymorphism (RFLP) of the amplicons was performed with the restriction enzymes *HinfI* and *TaqI* (Fast Digest, Thermo Scientific), used individually at a final concentration of 0.5 U/µl and temperatures of 65 °C and 37 °C, respectively, for 10 min. Electrophoresis in 3% agarose gel was carried out to visualize the banding patterns of the larvae studied, in order to determine their species according to D'Amelio et al. (2000) and Romero et al. (2014). Some larvae showed a mixed banding pattern between *A. simplex s.s.* and *A. pegreffii* with one or other restriction enzyme and were thus classified, for the purposes of this study, as hybrid type I larvae. The digestion controls with *TaqI* of the DNA amplicon of *A. simplex s.s.* produced 3 fragments of 430, 400 and 100 bp while those of *A. pegreffii* produced 3 bands of 400, 320 and 150 bp. When digestion was performed with *HinfI*, the fragments were of 620, 250 and 80 bp for *A. simplex s.s.* and 370, 300 and 250 bp for *A. pegreffii*. In the case of the hybrids of the two species, the banding pattern was the sum of the bands of both species for the enzyme in question. For *A. physeteris*, 3 bands of 300, 280 and 140 were obtained with *TaqI* and another 3 of 380, 290 and 270 for *HinfI*.

2.3. Epidemiological parameters and statistical analysis

The epidemiological parameters prevalence (P), mean intensity (MI) and mean abundance, defined by Bush et al. (1997), were calculated and compared using the free software Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005) to address the notoriously left-biased frequency distributions of parasites, based on the theoretical work of Rózsa et al. (2000). The differences in prevalence were evaluated using Fisher's exact test. A bootstrap 2-sample t-test (with 20000 repetitions) was used to compare mean intensities and mean abundances. Student's *t* test was used for the statistical comparison of length, weight and condition factor of the fish.

3. Results

3.1. Host

The length range of the 140 blue whiting examined was 14.3-30.2 cm, weight range was 24.3-251.1 g and condition factor (CF) range was 0.66-1.06 (Table 2). The relationship between weight (W) and length (L) showed a potential line with an exponent close to 3 ($W = 0.0072 \times L^{3.0567}$; coefficient ± 0.0008 and exponent ± 0.0362, $R^2 = 0.981$), showing a relationship generally considered in the literature as cubic (Fulton, 1904; García et al., 1987; Nash et al., 2006). Although length and weight were lower in autumn, CF values were similar throughout the three seasons of sampling (Table 2).

3.2. Epidemiological parameters

Total prevalence of L3 of *Anisakis* spp. was 29.3%, with significant variation between spring and autumn (Table 2), being higher in spring, independently of the year. Of the 74 larvae collected from fish, 61 % were type I and the remaining 39% type II. Three fish (2.1%) showed larvae in muscle tissue. Table 3 shows the epidemiological parameters according to *Anisakis* L3 morphotype and season (excluding spring 2016) in which the fish were caught. It can be seen that both the total prevalence by morphotype and its seasonal variation within each morphotype was statistically significant, being greater in spring than autumn ($p<0.001$).

When the fish were studied according to season of capture and length groups, excluding spring 2016, (Table 4), it was observed that prevalence increased with length, regardless of *Anisakis* type, especially in spring, which is when the largest specimens were captured. The prevalence was still significantly different even when fish of the same length class (17.7-22.3 cm) from autumn 2016 and spring 2017 were compared (Table 4). When extreme lengths (the largest and smallest fish) were removed from this length class so that neither group (autumn 2016 and spring 2017) showed statistical differences in weight, length and CF, significant differences were still observed ($P<0.03$) in prevalence and abundance, at least in *Anisakis* type I. Furthermore, comparison of the size of infected fish with uninfected fish revealed that both length and weight were significantly higher in the former, but

without affecting the CF of the fish (Table 5). In order to make an approximate estimation of the age at which the blue whiting of the study area first became infected, the average length of fish with a single larva was determined. For *Anisakis* type I, the average length of the blue whiting was 22.0±3.6 cm (n=28) and for *Anisakis* type II, 23.0±4.5 cm (n=8), corresponding to a fish age of 2.3 and 2.8 years respectively (García et al., 1987).

3.3. Molecular identification of Anisakis *larvae by PCR-RFLP*

Of the 45 *Anisakis* type I larvae collected, 91.1% were molecularly identified as *A. pegreffii*, 2.2% as *A. simplex s.s.*, while the remainder (6.7%) showed a hybrid PCR-RFLP band pattern with one of the restriction enzymes employed (*TaqI* or *HinfI*). All the type II larvae analyzed (27 of 29) were molecularly identified as *A. physeteris*. Four larvae (5.4% of all the larvae) were found in muscle after pepsin digestion, all type I: two larvae (1 *A. simplex s.s.* and 1 *A. pegreffii*) in one fish, while two fish each harboured 1 larva of *A. pegreffii* in their muscle.

3.4. Molecular epidemiological parameters

The epidemiological parameters for infection by the *Anisakis* species molecularly identified in the fish are shown in Table 6. Prevalence of *A. pegreffii* (19.3%) was almost double that of *A. physeteris* (10.0%) ($p<0.05$), while being very low for *A. simplex s.s.* (0.7%). Only 2.1% of the fish were infected with *A. pegreffii/A. simplex s.s.* hybrid larvae. The coinfection prevalence was 3.6%: 3 blue whiting harboured larvae of *A. pegreffii* and *A. physeteris*, one fish hosted 1 hybrid larva and 1 *A. physeteris* larva and one blue whiting hosted larvae of *A. pegreffii* (10), *A. simplex s.s.* (1) and *A. physeteris* (1).

4. Discussion

Since 1955 (Poljanskij, 1955) numerous studies on anisakid infection of blue whiting from the NE Atlantic Ocean have been carried out. Identification was based on morphological characteristics with different authors reporting L3 of *Anisakis* sp., *Anisakis* type I or *Anisakis simplex* (see Table 1). When the number of fish examined was ≥50, prevalence was always very high (>53%; see Table 1). In addition to *Anisakis*, L3 of *Hysterothylacium aduncum* have often been reported. Other anisakids occasionally

147 found were L3 of *Contracaecum* sp., *C. osculatum*, *Pseudoterranova decipiens*, and *Hysterothylacium*
148 sp. and L4 and adults of *H. aduncum* (references in Table 1, and Karasev, 1990). In the Mediterranean
149 Sea, the first studies were carried out by Orecchia et al. (1989) in Italian waters, and, for the first time,
150 the molecular identification of *Anisakis* in blue whiting was performed, detecting *A. simplex* A (=*A.*
151 *pegreffii*, P=62.1%) and *A. physeteris* (P=2.7%). Valero et al. (2000) molecularly identified *A. pegreffii*
152 (P=6.7%) in waters of the northern Alboran Sea. The identification of morphotype II larvae was
153 morphological and reported as *A. physeteris* (P=2.7%). About 300 km north of the area studied in this
154 work, Osanz Mur (2001) detected only *Anisakis* type I in blue whiting (P=22.6%). Here, similar
155 prevalence values (P=22.9%) were reported for morphotype I (20.7% for *A. pegreffii*, 2.1% hybrids *A.*
156 *pegreffii/A. simplex s.s.*, and 0.7% *A. simplex s.s.*), but morphotype II (=*A. physeteris*) was also identified
157 (P=10.0%). Finally, in the coastal waters of Sardinia a P>60% for *Anisakis* type I (=*A. pegreffii* and a few
158 hybrids *A. pegreffii/A. simplex s.s.*) and P= 5-10% for morphotype II (=*A. physeteris*) larvae were
159 reported (Angelucci et al., 2011; Meloni et al., 2011; Piras et al., 2014; Tedde et al., 2011).

160 It is accepted that both the fishing area and the size of the fish are risk factors associated with infection
161 by anisakids (Adroher et al., 1996; Molina-Fernández et al., 2015; Rello et al., 2009; and others). In this
162 sense, the area analyzed (western WM) showed a low abundance (Tables 1 and 2) of larvae of *Anisakis*
163 type I in blue whiting with which the prevalence was relatively low compared to other areas surveyed
164 in the Mediterranean Sea (eastern WM) and the NE Atlantic Ocean. There were also notable
165 differences regarding *Anisakis* type II, whose presence in the blue whiting of the surveyed areas of the
166 NE Atlantic Ocean is rare (see Table 1; Adroher and Benítez, in Bay of Biscay, N Spain, unpublished
167 data). In contrast, in the surveyed areas of the WM, the prevalence of *Anisakis* type II is more or less
168 uniform (P= 5-10%) and identified as *A. physeteris*, although *A. paggiae* has also been described
169 (Romero et al., 2014).

170 Regarding the size (age) of the fish, several authors have shown that the prevalence of anisakid
171 infection increases with the length (age) of the blue whiting (Madrid et al., 2012; Valero et al., 2000;
172 this report). García et al. (1987) reported that the longer, sexually mature blue whiting migrate to

greater depths from August to February to spawn, so they are not caught by trawlers in this area of the western WM, being replaced by the blue whiting > 11 cm that climb to the depths of capture. The fact that the larger blue whiting are caught in the spring could explain why a higher prevalence is detected in that season (Gómez-Mateos et al., 2016; Madrid et al., 2012; Valero et al., 2000; this report). However, the comparison of fish of the same length (age), weight and CF between seasons results in a statistically significant difference, at least for Anisakis type I, and thus other unknown factors, in addition to age, may have been affecting the seasonal prevalence of infection. The formula to obtain the age of the WM blue whiting, after García et al. (1987), attributes a mean age of 1.4 years to those not infected, of 2.4 years to those infected with *Anisakis* type I (2.3 y for fish hosting one larva) and of 3.8 years to those infected with *Anisakis* type II (2.7 y for fish hosting one larva). These data suggest that blue whiting are not usually infected with *Anisakis* until they are over 1 year old and that infection by type II takes place later than that by type I. This may be related to diet but also to higher food intake in larger fish, since, from the age of 1 year they primarily consume euphausiids and small fish (potential intermediate/paratenic hosts) and there is a significant correlation between abundance of prey in the diet and in the environment (Macpherson, 1978). However, this author reported that cephalopods, paratenic hosts of *A. physeteris* (Mattiucci and Nascetti, 2008; Orecchia et al., 1989), generally became part of the diet of blue whiting of the WM on the latter attaining a length of 24 cm (~3.2 y), which would, at least partially, explain the results obtained. Angelucci et al. (2011) detected *Anisakis* type II larvae in the squid *Todarodes sagittatus* (P=20%) in Sardinian waters, and Picó-Durán et al. (2016) in the squid *Illex coindetii* (P=1.6%) in Spanish Mediterranean waters. Furthermore, Valero et al., (2000) observed that the prevalence of *A. physeteris* in blue whiting of ≥23 cm from the northern Alborán Sea (WM) was three times that found in smaller specimens. The CF of fish does not seem to be affected by the *Anisakis* infection (Tables 2 and 5). Controversially, some authors have suggested that CF is not only affected when the parasitic intensity is high, but also by factors such as the season or the age (length) of the fish (see Rohde, 1984, and references therein).

Finally, although some *Anisakis* larvae were found in muscle tissue, the risk of human infection seems low as they were few in number in the surveyed zone and the blue whiting is not usually eaten raw. In

any case, it should not be forgotten that ≥1 million tons of this fish are sold every year, which means that the possibility of cases must be considered by the health authorities. Moreover, although several authors have not detected significant migration of the larvae from the visceral cavity to the muscle in the 72 h after capture (Smith, 1984; Wootten and Smith, 1976; Zubchenko et al., 1980), Madrid et al. (2012) showed a statistical relationship between the number of *Anisakis* larvae in blue whiting muscle and the time since capture (up to 7 days). Furthermore, other authors have described a greater presence of *Anisakis* type I larvae in blue whiting muscle in other fishing zones in both Atlantic and Mediterranean waters (see Table 1) Consequently, suitable thermal treatment is required before consuming any fish product which may be infected with *Anisakis*.

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Compliance with ethical standards.

Conflicts of interest: none.

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Table 1.- *Anisakis* infection in blue whiting, *Micromesistius poutassou*. Published surveys.[a]

Reference	Hosts analyzed	Origin-FAO fishing zone	Prevalence of L3 *Anisakis* spp.	Mean intensity (range)	Parasites in muscle P; MI[b] (range)	Species
Poljanskij, 1955	5	Barents Sea-27.I	40%	1		*Anisakis* sp.
Berland, 1961	12	Hordaland, W Norway-27.Iva	58.3%			*Anisakis* sp. type I
	3	Tromsø, N Norway-27.Iia	100%			*Anisakis* sp. type I
Raitt, 1968[c]	20	Faroe Islands-27.Vb	100%	14	Yes	*Anisakis* sp.
	20	W Scotland-27.Via	25%	1		*Anisakis* sp.
Reimer et al., 1971	80	W Ireland-27.Va	67.8%			*A. simplex*
	100	NW Iceland-27.Va	70%			*A. simplex*
Wootten and Smith, 1976	610	W Scotland-27.VIIb	97.4%	54.9	MI=5.2-18.6	*Anisakis* type I
Richards, 1977	?	W Scotland-27.VIIb	13-24%			*Anisakis* sp.
	?	Faroe Islands-27.Vb	27-32%			*Anisakis* sp.
Bussmann and Ehrich, 1978	4100	Faroe Islands-27.Vb	High		MI=3.5-7.2	*Anisakis* sp.
Grabda, 1978	50	N Ireland--27.Via	98%	(7-679)	P=88%; MI=5.6 (1-20)	*Anisakis* sp.
Smith and Wootten, 1978	980	W and N Scotland-27.Via	92.3%	(1-583)	Yes	*Anisakis* sp.
MacKenzie, 1979	74	W and N Scotland-27.Via	74.3%		Yes	*Anisakis* sp.
Højgaard, 1980	458	Faroe Islands-27.Vb	High		P=83.6%	*A. simplex*
Kusz and Treder, 1980	30	Faroe Islands-27.Vb	100%	60.0 (1-239)	P=63.3%; MI=4.3 (1-19)	*A. simplex*
Schultz et al., 1980	829	Norwegian Sea-27.Iia	Yes		Yes	*Anisakis* sp.
Zubchenko et al., 1980	175	Norwegian Sea-27.Iia	Yes		P=99.4%; MI=5.5 (1-34)	*Anisakis* sp.
Giedz, 1981	647	Celtic Shelf-27.Iva	Yes		P=27.0%	*Anisakis* spp.
	1235	Faroe Islands-27.Vb	Yes		P=33.3%	*Anisakis* spp.
Karasev et al., 1981	269	Norwegian Sea-27.Iia	99.2%	43.8	P=37.9%; MI= 1.5	*Anisakis* sp.
Dumke, 1988	2107	North Atlantic-27.IIa,IIb, IIIa, Vb, XIVa	Yes		P=54.1-99.5%	*Anisakis* sp.
Orecchia et al., 1989	487	Italian waters, Mediterranean Sea 37.1.3 and 37.2	Yes			*A. pegreffii** (P=62.1%) *A. physeteris** (P=2.7%)
Sanmartín Durán et al., 1989	67	W Galicia, NW Spain-27.VIIIc, Ixa	62.3%	5.8		*Anisakis* type I
Cuéllar et al., 1991	40	Valencia Gulf, E Spain-37.1.1	30.3%	(1-10)		*Anisakis* type I
Pereira-Bueno, 1992	42	Bilbao (Spain) fishmarket	88.1%	33.5	P=52.4%; MI=21.9	A. *simplex*
Ruiz-Valero et al., 1992	299	Granada (Spain) fishmarket	Yes	13.3	P=29.1%; MI=7.0 (1-56)	*A. simplex* (P=67.9%) *Anisakis* sp. (P=2.3%)
Køie, 1993	10	Faroe Islands-27.Vb	100%			*A. simplex* s.l.
López Giménez and Castell Monsalve, 1994	82	Castilla La Mancha (Spain) fishmarkets	29.3%			*Anisakis* spp.
Sanmartín et al., 1994	179	W Galicia, NW Spain-27.VIIIc, IXa	67.0%	5.9	P=20.0%	*A. simplex*
Viu et al., 1996	62	Zaragoza (Spain) fishmarket	85.5%	7.1 (1-61)	P=9.4%	*Anisakis* type I *Anisakis* type II
Pereira-Bueno and Ferre-López, 1997	11	Castilla y León (Spain) fishmarkets	63.6%			*A. simplex*
Manfredi et al., 2000	345	Ligurian Sea-37.1.3	32.4-65.5%	1.5-2.3		*A. simplex*
Valero et al., 2000[d]	301	Motril Bay, N Alboran Sea-37.1.1	9.0%	1.2 (1-4)	P=0.3%; MI=1.0	*A. pegreffii*# (P=6.7%) A. *physeteris* s.l. (P=2.7%)

Ojeda-Torrejón et al., 2001	390	Gulf of Cadiz, SW Spain-27.IXa	53.6%		Yes	*A. simplex*
Osanz-Mur, 2001	562	Tarragona waters, E Spain-37.1.1	25.1%	1.3 (1-3)	Yes	Anisakids
Silva and Eiras, 2003	65	W Portugal-27.Ixa	93.8%	14.3 (1-89)	Yes	*Anisakis* sp.
Fernández et al., 2005	400	Rias Baixas, Galicia, NW Spain-27.IXa	99.3%	11.1	Yes	*A. simplex* s.l. *A. physeteris* (P=0.25%; La=0.04%) [e]
Martín-Sánchez et al., 2005	401	Mediterranean coasts of Spain-37.1.1	9.1%			A. *pegreffii** (La=59.3%) A. *simplex* s.s.* (La=18.5%) Hybrids[f]* (La=18.5%) New genotype type I (La=3.7%)
		Atlantic coasts of Spain-27.VIIIc, Ixa	81.7%			A. *pegreffii** (La=20%) A. *simplex* s.s.* (La=66.7%) Hybrids (La=13.3%)*
Cruz et al., 2007	238	Matosinhos, NW Portugal-27.Ixa	77.7%	5.8 (1-122)	P=27.7%; MI=2.0	*Anisakis* sp.
Mattiucci and Nascetti, 2007	?	Mediterranean Sea-37	48.5%	(1-12)		A. *pegreffii**
Chía et al., 2010	119	NW Spain-27.VIIIc, Ixa	100%	55.9	P=37.0%; MI=24.2 (1-327)	*Anisakis* type I
Angelucci et al., 2011	16	Sardinia-37.1.3	87.5%	10	P=62.5%; MI=1.4	*Anisakis* type I (P=81.2%) *Anisakis* type II (P=12.5%)
Meloni et al., 2011	17	Sardinia-37.1.3	82.4%			A. *pegreffii** (La=90.6%) A. *physeteris** (La=1.3%) Hybrids* (La=8.1%)
Tedde et al., 2011	57	N Sardinia-37.1.3	61.4%	3.9 (1-50)	P=10.5%	*A. pegreffii**# A. *physeteris** (P=5.3%)
Madrid et al., 2012	169	NE Atlantic-27.VIIIc, Ixa	78%	(1-95)	P=39%	*A. simplex* s.l.
	115	W Mediterranean Sea-37.1	19%	(1-219)	P=7%	*A. simplex* s.l.
Romero et al., 2013[d]	?	Mediterranean coast of Spain-37.1.1	Only surveyed type I			*A. pegreffii** (La=64%) *A. simplex* s.s.* (La=19%) Hybrids* (La=17%)
	?	Atlantic coasts of Iberian Peninsula-27.VIIIc, Ixa	Only surveyed type I			*A. pegreffii** (La=28%) *A. simplex* s.s.* (La=49%) Hybrids* (La=23%)
Romero et al., 2014[d]	?	Mediterranean coast of Spain-37.1.1	Only surveyed type II			A. *physeteris*[F]* (La=55%), *A. paggiae** (La=45%)
	?	Atlantic coasts of Iberian Peninsula-27.VIIIc, IXa	Only surveyed type II			A. *physeteris** (La=45%), *A. paggiae** (La=50%) *A. brevispiculata** (La=5%)
Piras et al., 2014	57	Gulf of Asinara, N Sardinia -37.1.3	High	(1-50)	P=14%; MI=1	*A. pegreffii**# (P=>66.7%) *A. physeteris** (P=10.5%)
Gómez-Mateos et al., 2016	100	Gulf of Cadiz, SW Spain-27.IXa	82%	16 (1-328)	P=38%	A. *simplex* s.s.*# (La=50%) A. *pegreffii**# (La=42.7%) Hybrids*# (La=7%) *A. typica** (La=0.25%)
This report	140	Villajoyosa port (E Spain)-37.1.1	29.3%	1.8 (1-12)	P=2.1%; MI=1.3 (1-2)	*A. pegreffii**# (P=19.3%; La=55.4%) *A. simplex* s.s.*# (P=0.7%; La=1.3%)

						Hybrids* (P=2.1%; La=4.1%) A. *physeteris** (P=10.0%; La=39.2%)

[a]In some surveys the occurrence of other anisakids has been studied: *Hysterothylacium aduncum* L3 has been detected with high prevalence, and less frequently L4 and adults of *H. aduncum*. Also, the presence of L3 of *Hysterothylacium* sp., *Contracaecum* sp., *C. osculatum*, and *Pseudoterranova decipiens* has been occasionally reported. See Karasev (1990).
[b]P, prevalence; MI, mean intensity.
[c]Data from Dr. Kabata.
[d]Data calculated from the reference.
[e]La= percentage of *Anisakis* larvae.
[f]Hybrids: recombinant genotype of *A. pegreffii* and *A. simplex s.s.*
*Molecular identification.
#Also detected in muscle.

418 Table 2.- Epidemiological parameters of infection by *Anisakis* spp., total and by season, in blue whiting sampled
419 on the Mediterranean coast of eastern Spain.

Parameter	Total	Spring 2016[#]	Autumn 2016	Spring 2017[#]
N blue whiting	140	13	81	46
Length ±SD (range)	20.4±3.6 (14.3-30.2)	25.5±3.3*** (21.1-30.2)	18.2±1.8 (14.3-22.3)	22.9±3.0*** (17.7-28.4)
Weight ±SD (range)	80.0±45.7 (24.3-251.1)	155.5±59.4*** (84.9-251.1)	52.8±17.0 (24.3-101.9)	106.6±36.2*** (45.7-173.5)
Condition Factor ±SD (range)	0.86±0.06 (0.66-1.06)	0.90±0.06* (0.77-0.99)	0.85±0.05 (0.66-0.98)	0.86±0.07[ns] (0.71-1.06)
Prevalence CI 95%	29.3 (22.1-37.5)	46.2** 22.4-74.0	9.9 4.6-18.4	58.7*** 43.4-71.9
Mean Intensity (range) CI 95%	1.80 (1-12) 1.39-2.73	2.67[ns] (1-6) 1.33-4.17	1.00 (1) Uncertain	1.85[ns] (1-12) 1.30-3.22
Mean Abundance CI 95%	0.53 0.36-0.83	1.23[ns] 0.38-2.54	0.10 0.04-0.16	1.09* 0.70-1.98

420 Weight in g; length in cm. SD=standard deviation. Prevalence=100·F/N, mean intensity=A/F, mean
421 abundance=A/N; where N is the total number of fish, F is the number of infected fish, and A is the number of
422 larvae. CI: confidence interval. Comparison of seasonal prevalence showed $p<0.001$. Comparison between spring
423 2016 and spring 2017 not significant for all the epidemiological parameters compared, except length and weight
424 ($p<0.05$).
425 [#]Shows the pairwise comparison spring 2016-autumn 2016 and autumn 2016-spring 2017: [ns]not significant;
426 *$p<0.05$; **$p<0.005$; ***$p<0.0001$.

427 Table 3.- Epidemiological parameters of infection by season for the two *Anisakis* L3 morphotypes in blue whiting.

Parasite		Total	Autumn 2016	Spring 2017
Anisakis type I	Prevalence	22.9	8.6	50.0
	CI 95%	16.3-30.7	4.1-17.1	35.8-64.2
	Mean Intensity (range)	1.41 (1-11)	1.00 (1)	1.57 (1-11)
	CI 95%	1.06-2.63	Uncertain	1.09-2.96
	Mean Abundance	0.32	0.09	0.78
	CI 95%	0.21-0.61	0.02-0.15	0.48-1.59
Anisakis type II	Prevalence	10.0**	$1.2^{\$}$	19.6**
	CI 95%	6.0-16.3	0.07-6.6	10.3-33.6
	Mean Intensity (range)	2.07^{ns} (1-6)	1.00 (1)	1.56^{ns} (1-4)
	CI 95%	1.36-3.00	Uncertain	1.11-2.33
	Mean Abundance	0.21^{ns}	0.01*	0.30^{ns}
	CI 95%	0.10-0.37	0.00-0.04	0.13-0.59

428 Data of fish caught in spring 2016 not included. CI: confidence interval.
429 Statistical comparison between morphotypes: $^{\$}p<0.07$ (Fisher's exact test) but $p<0.04$ (Exact unconditional test);
430 *$p<0.05$; **$p<0.005$. Comparison of the seasonal variation of prevalence within each morphotype shows $p<0.001$
431 in both cases.

432

Table 4.- Variation of the prevalence of *Anisakis* in blue whiting according to length groups and season of capture.[a]

Blue whiting	Prevalence (F/N)[b] *Anisakis* spp.		Prevalence (F/N) *Anisakis* type I		Prevalence (F/N) *Anisakis* type II	
Length class (age)[c]	Autumn 2016	Spring 2017	Autumn 2016	Spring 2017	Autumn 2016	Spring 2017
<17.7 cm (<1 y)	9.7 (3/31)	--	6.5 (2/31)	--	3.2 (1/31)	--
17.7-22.3 cm[d] (~1-2.5 y)	10.0 (5/50)	57.1*** (12/21)	10.0 (5/50)	42.9** (9/21)	0 (0/50)	14.3* (3/21)
>22.3 cm (>2.5 y)	--	60.0 (15/25)	--	56.0 (14/25)	--	24.0 (6/25)

[a]Fish caught in spring 2016 not included as cannot be classified into significant groups due to their low number.
[b]Where N is the total number of fish and F is the number of infected fish.
[c]Approximate age class according to García et al. (1987).
[d]This class of length covers the fish included between the minimum size of the spring sample and the maximum size of the autumn one.
Statistical comparison between seasons: *p<0.05; **p<0.005; ***p<0.001.

Table 5.- Epidemiological infection parameters of blue whiting by *Anisakis* morphotype.

Parameters		*Anisakis* spp.	*Anisakis* type I	*Anisakis* type II
Fish Length[a] ±SD	U	19.5±2.9	19.9±3.4	19.9±3.1
	I	22.7±4.1***	22.2±3.7**	25.3±4.4***
Fish Weight ±SD	U	68.0±33.2	74.6±45.0	72.7±36.2
	I	109.0±57.9***	98.5±44.1*	146.5±67.1**
Fish Condition Factor ±SD	U	0.86±0.06	0.86±0.06	0.86±0.06
	I	0.86±0.07 ^{ns}	0.85±0.06 ^{ns}	0.84±0.08 ^{ns}

Weight in g; length in cm. SD=standard deviation. Abbreviations: U, uninfected fish; I, infected fish.
Statistical analysis to compare morphometrical fish parameters between uninfected and infected fish (Student's *t* test): $^{*}p<0.05$; $^{**}p<0.007$; ***$p<0.0005$; ^{ns}not significant.
Comparison between morphotypes (Student's *t* test): Length ($p<0.04$) and weight ($p<0.03$) are statistically different in infected fish but the same in uninfected fish ($p>0.7$). CF, ns.
[a]According García et al. (1987): mean age of 1.4 years to fish not infected (19.5 cm), of 2.4 years to those infected with *Anisakis* type I (22.2 cm) and of 3.8 years to those infected with *Anisakis* type II (25.3 cm).

451 Table 6.- Epidemiological infection parameters of blue whiting by species of *Anisakis* genetically identified.

Parasite	Prevalence CI 95%	Mean Intensity (range) CI 95%	Mean Abundance CI 95%
A. physeteris	10.0 6.0-16.3	2.07 (1-6) 1.36-3.00	0.21 0.10-0.37
A. pegreffii[a]	19.3* 13.5-26.7	1.44 [ns] (1-10) 1.07-2.67	0.28 [ns] 0.18-0.54
A. simplex s.s.	0.70 0.04-3.81	1 (1) Uncertain	0.01 0.00-0.02
Hybrids[b]	2.1 0.6-6.3	1 (1) Uncertain	0.02 0.00-0.04

452 CI: confidence interval.

453 [a]Statistical analysis to compare epidemiological parameters *A. physeteris* vs. *A. pegreffii*: [*]p<0.05; [ns]not
454 significant.

455 [b]Hybrids: these are the larvae showing a hybrid PCR-RFLP band pattern which was the sum of the bands of *A.*
456 *simplex s.s.* and *A. pegreffii* for one of the restriction enzymes (i.e. *HinfI* or *TaqI*).