



# Anisakis infection in anchovies (*Engraulis encrasicolus*) from Iberian waters, southwestern Europe: Post-mortem larval migration

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## SUMMARY

The *Anisakis* larvae presence in fish for human consumption is a health risk that needs to be monitored. The anchovy is a fish that is highly appreciated by consumers and that can harbour *Anisakis*. It is thus necessary to periodically evaluate the presence of anisakid larvae in them. So, anchovies from Iberian Peninsula coasts were analysed. Fish examination for macroscopic nematodes showed L3s of both *Anisakis* type I and *Hysterothylacium aduncum*. The *Anisakis* prevalence varies with the catching area and the fish size. The muscle prevalence was 7.45% (mean intensity 1.75; range 1–5). Molecular analysis showed 110 *A. simplex* s.s. (17 in muscle), 22 *A. pegreffii* (3) and 7 hybrid genotype individuals (1). Considering that most of the Iberian Peninsula coasts are a sympatry area between these two *Anisakis* species, it has been observed that *A. simplex* s.s./*A. pegreffii* ratio increases from south to north in a clockwise direction. Also, 19 larvae were detected on the fish surface from the Bay of Biscay, indicating the ability of these larvae to migrate after the fish death. The *A. simplex* s.s./*A. pegreffii* larvae proportion found on the anchovy surface is similar to the found in viscera and lower than in muscle, suggesting that most of the larvae migrating to the surface must have come from the visceral package. This confirms the importance of removing fish viscera immediately after capture, for those fish species where this is possible. As both species cause anisakiasis/anisakidosis, these data show a real risk to human health, especially in dishes highly prized in Mediterranean countries prepared with raw or semi-raw anchovies.

## 1. Introduction

The ingestion of *boquerones en vinagre* is considered to be one of the most frequent ways of acquiring anisakiasis/anisakidosis in Spain and probably also in neighbouring countries such as France and Italy where anchovies are also consumed marinated (Bao et al., 2019). Although in Portugal, it is less common, the recent report of new cases in this country and the increased tendency to prepare raw, pickled (lemon/vinegar) or undercooked fish dishes (Baptista-Fernandes et al., 2017; Golden et al., 2023, 2022; Santos et al., 2022) makes it advisable to monitor anisakid infection in anchovies, but also in other more commonly consumed fish (Golden et al., 2022).

The anchovy *Engraulis encrasicolus* (Clupeiformes: Engraulidae) is a small highly-prized pelagic fish that inhabits the coastal areas of the Northeast Atlantic, as well as the coasts of the Mediterranean, including the Black Sea. It tends to move northwards and towards surface waters

in summer, retreating and descending in winter (Frimodt, 1995). Anchovies feed on planktonic organisms and spawn from April to November, with peaks usually in the warmer months (Frimodt, 1995). It is marketed in many different forms, especially fresh, but also dried, smoked, canned, frozen, and even in preparations such as anchovies in vinegar (*boquerones en vinagre*, *alici marinate*, ...) where the presence of *Anisakis* larvae has been detected, probably due to the lack of parasite monitoring in the processing (Smaldone et al., 2020) or fraudulent practices (see newspaper *Granada Hoy*, news July 10th, 2020, [https://www.granadahoy.com/costa\\_tropical/Clausuran-empresa-Costa-boquerones-anisakis\\_0\\_1481552048.html](https://www.granadahoy.com/costa_tropical/Clausuran-empresa-Costa-boquerones-anisakis_0_1481552048.html); Agencia Española de Seguridad Alimentaria y Nutrición, 2020).

Anisakiasis/anisakidosis is acquired by ingesting viable third stage (L3) larvae of anisakids, mainly *Anisakis simplex* s.l. (>97% of cases), when consuming fish and/or squids containing them, as they are intermediate/paratenic hosts of these parasitic nematodes. Furthermore,

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this ingestion of viable larvae may sensitise the individual leading to the development of allergy to *Anisakis* (Audfćana et al., 2002). The number of cases of anisakiasis worldwide is increasing for several reasons (Adroher-Auroux and Benítez-Rodríguez, 2020): the awareness of health workers and the development of more effective diagnostic techniques; the increased consumption of exotic dishes made with raw or undercooked fish; and, at least in some areas, the increased prevalence and/or intensity of anisakids in some fish species and even in cetaceans (ICES, 2002; McClelland et al., 2000; Podolska and Horbowy, 2003; Pons-Bordas et al., 2020).

Even with improved diagnosis, a significant underdiagnosis has been suggested, especially in countries with high per capita fish consumption (Adroher-Auroux and Benítez-Rodríguez, 2020; Bao et al., 2017; Herrador et al., 2019; Seal et al., 2020; Shamsi and Sheorey, 2018). Worldwide, the prevalence is estimated at 0.32 cases/100,000 inhabitants (Orphanet, 2020), which is about 25,000 cases, most of which occur in Asia, especially in Japan, where about 500 cases were diagnosed annually (Suzuki and Murata, 2011), although with probable underdiagnosis, as it is currently estimated at almost 20,000 cases (Sugiyama et al., 2022). About 500 cases/year are diagnosed in Europe (Arizono et al., 2012), of which >150 are in Spain (Audfćana et al., 2002). However, published estimates, based on the use of models, put the number of cases at 8000 per year in this country from anchovy consumption alone (Bao et al., 2017) and between 10,000 and 20,000 if calculated from hospital data on anisakiasis diagnosis from any source of infection (Herrador et al., 2019). These data highlight the high underdiagnosis that still exists in anisakiasis-aware countries. Accordingly, it is very likely that in other countries with high per capita fish consumption associated with increased tendency to consumption of raw or undercooked fish, and where cases of anisakiasis are scarcely diagnosed (such as Portugal and others), underdiagnosis is higher.

Given the importance of anchovies as a source of infection in the countries where they are consumed (Bao et al., 2017) and the opinion of the EFSA expert panel encouraging the scientific community to implement a continuous survey on *Anisakis* presence in fish intended for human consumption as one of the main preventive action possible (EFSA-BIOHAZ, 2010), we have carried out an epidemiological survey in the fishmarkets of Granada, a city in southern Spain, to determine the epidemiological parameters of anisakid infection and to determine the

factors that may lead to an increased risk of anisakiasis for the population through the consumption of this fish from the seas off the coasts of the Iberian Peninsula, in Southwestern Europe (see Fig. 1).

## 2. Material and methods

### 2.1. Host and parasites

The anchovies that arrive at the market in Granada come from the coasts of the Iberian Peninsula: from the Bay of Biscay (port of Ondarroa, Northern Spain, area FAO 27-ICES VIIIb), Portugal, western coast (port of Figueira da Foz; Atlantic western coast of Iberian Peninsula, area FAO 27-ICES IXa), Gulf of Cádiz (ports of Punta Umbria, Isla Cristina and Cádiz; Atlantic coast of Southern Spain, area FAO 27-ICES IXa), Alboran Sea (ports of Fuengirola and Málaga; Mediterranean coast of Southern Spain, area FAO 37.1.1), and Balearic Sea (ports of Altea and València; western Mediterranean Sea, Mediterranean coast of Eastern Spain, area FAO 37.1.1).

Between October 2020 and March 2021, 161 anchovies, *Engraulis encrasicolus*, were acquired from fishmarkets in Granada (Southern Spain), noting the area of capture and port of landing, and transported in ice to the laboratory. In the laboratory, each fish was weighed and measured before being dissected for the detection and isolation of nematodes observable to the naked eye in the visceral cavity. After this operation, to detect nematodes hidden from view, viscera and musculature were separated and subjected independently to pepsin digestion at pH 2.0, at 37 °C (Huss and Drewes, 1989), with shaking, for 1 and 3 h, respectively (Buzo-Domínguez et al., 2021). After this time, the results of digestion were carefully examined for any other nematodes released by the digestive process from both viscera and musculature. In all cases, the detected nematodes were washed with saline (NaCl, 0.9% w/v). Once identified morphologically with the aid of light microscopy as *Anisakis* type I (Berland, 1961; Hartwich, 1974; Petter and Maillard, 1988; Smith and Wootten, 1984) or as *Hysterothylacium aduncum* L3 (Adroher-Auroux and Benítez-Rodríguez, 2021; Berland, 1991; Petter and Maillard, 1988), the nematodes were placed in a labelled vial, all necessary data noted and then frozen at –20 °C until prepared for molecular identification.



Fig. 1. Map of Iberian Peninsula. Area of investigation showing ports where anchovies were landed (●) and the fishmarkets sampled (○).

## 2.2. Molecular identification of isolated larvae by PCR-RFLP

Each larva, previously identified as *Anisakis* L3, was individually prepared for DNA isolation using the commercial RealPure kit following the manufacturer's instructions. The rDNA fragment corresponding to the ITS1–5.8-ITS2 sequence was amplified using the NC5 (forward) and NC2 (reverse) primers described by Zhu et al. (1998). PCR conditions were as previously described (Buzo-Domínguez et al., 2021; Molina-Fernández et al., 2015). The expected size of the amplified fragment was around 1000 bp. Restriction fragment length polymorphism (RFLP) of the amplicons was then performed with *HinfI* and *TaqI* restriction enzymes (Fast Digest, Thermo Scientific), used individually at a final concentration of 0.5 U/μl and at temperatures of 37 °C and 65 °C, respectively, for 10 min. A 3% agarose gel electrophoresis was performed to visualise the banding patterns of the larvae studied, in order to determine the species in accordance with previous studies (D'Amelio et al., 2000; Pontes et al., 2005). Some larvae showed a mixed banding pattern between *A. simplex* s.s. and *A. pegreffii* with one or both restriction enzymes, being classified, for the purpose of this study, as hybrid larvae between both species. Likewise, 11 L3 of *H. aduncum*, morphologically identified, were subjected to PCR-RFLP (with the same procedure as *Anisakis* larvae), together with a control, to confirm specific identification of larvae (Kijewska et al., 2002; Tedesco et al., 2018).

## 2.3. Epidemiological parameters, statistical analysis, and analysis of risk factors of anchovy infection

The epidemiological parameters of prevalence (P), mean intensity (MI) and mean abundance (MA), as defined by Bush et al. (1997), were calculated and compared using the free software Quantitative Parasitology 3.0 (Reiczigel et al., 2019; Reiczigel and Rózsa, 2005) to address notoriously left-skewed parasite frequency distributions, based on the theoretical work of Rózsa et al. (2000). Differences in prevalence were assessed using Fisher's exact test. A 2-sample bootstrap *t*-test (with 20,000 replicates) was used to compare mean intensities and mean abundances. ANOVA was used for statistical comparison of fish length, weight and condition factor. Where this test was significant, Student's *t*-test was used for pairwise statistical comparison of the above host parameters. Values  $p \leq 0.05$  were considered significant.

To determine whether prevalence and intensity of infection by *Anisakis* (dependent variables;  $N = 101$ ) are influenced by catch area and/or body length of fish (independent variables), two additive general linear model analyses (GLMs), one logistic and one Gaussian, respectively, were performed. Significance level was estimated at 0.05. When catch area was significant, Tukey's honest significant difference (HSD) post-hoc tests were run to observe differences between fish catching areas. Additionally, to assess the differential effect of body length on infection parameters, independent GLMs for prevalence and intensity at each catch area were run. Host body weight was not considered in the analyses as it is strongly correlated with body length (Pearson's correlation coefficient = 0.96). Only areas where infection by *Anisakis* was detected (Bay of Biscay, Portugal and Gulf of Cádiz) were included, with Alboran Sea and Balearic Sea being excluded. Statistical analyses were conducted in R software, v. 4.1.0 (R Core Team, 2013) using the *car* (Fox and Weisberg, 2019) and *multcomp* (Hothorn et al., 2008) packages.

## 3. Results

### 3.1. Host and parasites

A total of 161 anchovies from 5 geographical areas of the Iberian Peninsula coasts were analysed. The mean total length (L)  $\pm$  SD (standard deviation) of the sample was  $12.9 \pm 1.7$  cm, while the mean total weight (W)  $\pm$  SD was  $13.14 \pm 5.54$  g. Fulton's condition factor (CF) was calculated by the formula  $CF = 100 \times W/L^3$ , where W = total weight (g) and L = total length (cm) with the mean =  $0.587 \pm 0.110$ . This CF is

considered an indicator of the general health of the fish (Monstad, 1990). The relationship between weight and length (Fig. 2) is considered to be cubic (Froese, 2006; Fulton, 1904) for isometric growth. In this case, the exponent is somewhat  $<3$ , resulting in the eq.  $W = 0.012 \times L^{2.714}$  (coefficient  $\pm 0.003$  and exponent  $\pm 0.101$ ,  $R^2 = 0.820$ ), although results vary when data are separated by catch areas resulting in allometric growth (results not shown). Comparison of anchovies from different areas showed significant differences (ANOVA,  $p < 0.0001$ ) in length, weight and CF. When the lengths of anchovies from the 3 areas showing *Anisakis* infection were compared (per pairwise; Table 1), they were shown to be different from each other (Student's *t*-test,  $p < 0.0001$ ).

Of the 101 anchovies sampled from the Atlantic coasts, 30 were infected with 139 L3 of *Anisakis*, all of type I (sensu Berland, 1961). The larvae were distributed as follows: 119 larvae in 18 anchovies from the Bay of Biscay, 17 in 10 from Portugal and 3 in 2 from the Gulf of Cadiz. None of the 60 Mediterranean anchovies analysed, both from the Balearic Sea and from the Alboran Sea, contained *Anisakis* larvae.

Of the *Anisakis* larvae found, 99 were in viscera, 21 in muscle and 19 on the surface of the anchovies. The prevalence of *Anisakis* in muscle was 7.45% with a mean intensity of 1.75 (range 1–5).

Figure 3 shows that in the sample of anchovies examined there is no presence of *Anisakis* spp. in those smaller than 13.6 cm ( $<1.4$  years), with the highest prevalence in those longer than 15.5 cm ( $>1.9$  years). The calculation of fish age from anchovy length is based on data published by Bellido et al. (2000). Furthermore, the data were grouped into three host length classes, as shown in Table 2, in order to give greater statistical consistency to their analysis.

L3 of *Hysterothylacium* spp. were only isolated from anchovies from the Atlantic coasts, with low prevalence and medium intensity in the Gulf of Cadiz (4.9%; 1) and Portugal (5%; 1) and high prevalence (95%) and medium intensity (7.3) in anchovies from Bay of Biscay. A total of 143 larvae were isolated and identified as L3 of *H. aduncum*.

### 3.2. Genetic identification of isolated larvae by PCR-RFLP

The 139 L3 of *Anisakis* type I collected from the anchovies were analysed by PCR-RFLP, revealing that there were 110 *A. simplex* s.s. (17 of them in muscle), 22 *A. pegreffii* (3 in muscle) and 7 individuals of hybrid genotype (1 in muscle) between the two previous species (4 for the two restriction enzymes used and 3 for 1 of them). By areas, in Bay of Biscay the proportion of larvae was 8.6:0.9:0.5 (*A. simplex* s.s.: *A. pegreffii*:hybrids); in Portugal it was 4.7:4.7:0.6; while in Gulf of Cadiz all larvae were *A. pegreffii*. In muscle, 17 *A. simplex* s.s. (15 in Bay of Biscay and 2 in Portugal), 3 *A. pegreffii* (1 in Bay of Biscay and 2 in Portugal) and 1 hybrid genotype (in Bay of Biscay), only for *TaqI*, were detected. In 9 anchovies from Bay of Biscay, 19 larvae were found on their surface, 17 *A. simplex* s.s. and 2 *A. pegreffii* (Table 3). Comparing

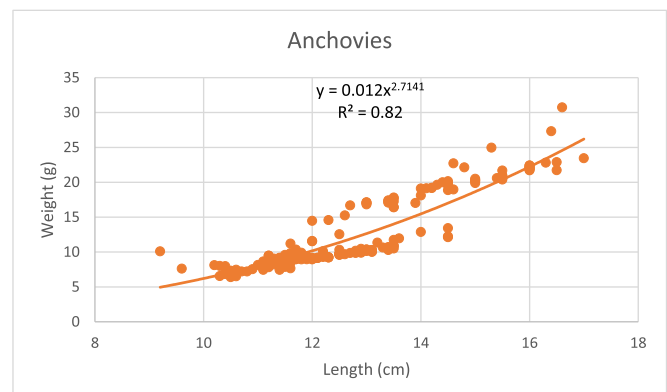
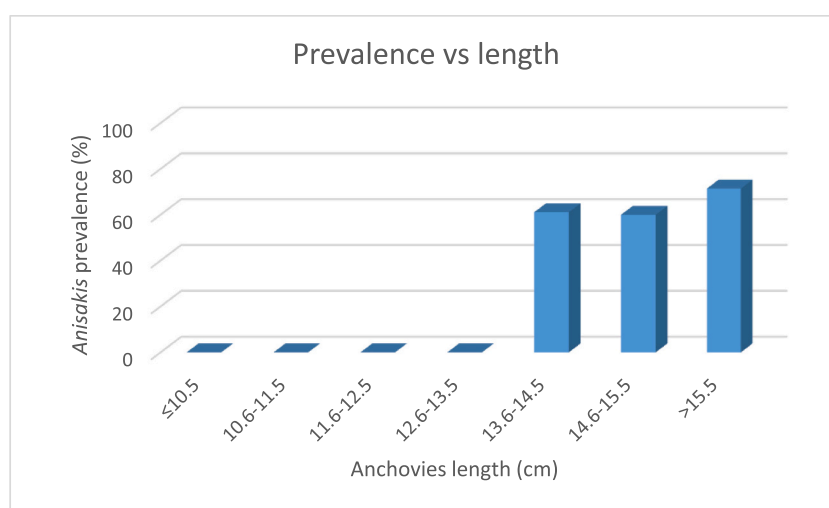


Fig. 2. Relationship between length and weight in all anchovies surveyed.

**Table 1**Host and epidemiological parameters of the anchovies parasitized by *Anisakis* spp. from the surveyed areas of Iberian waters.

Parameters	All areas	Bay of Biscay <sup>1</sup>	Portugal <sup>2</sup>	Gulf of Cádiz <sup>3</sup>	Alborán Sea	Balearic Sea
N° of fish	161	20	20	61	30	30
Mean length	12.9** (1.25 y)	14.7 (1.64 y)	15.8 (1.98 y)	11.8 (1.07 y)	12.0 (1.10 y)	12.9 (1.25 y)
±SD	±1.7	±0.9	±0.5	±1.2	±0.9	±0.8
(range)	9.2–17.0	13.4–16.6	15.0–17.0	9.2–14.5	9.6–13.4	11.5–14.5
Mean weight	13.14**	20.73	21.56	11.15	9.17	10.46
±SD	±5.54	±3.54	±0.95	±4.26	±0.73	±1.20
(range)	6.39–30.73	16.44–30.73	20.20–23.45	6.39–20.00	7.61–10.34	8.67–13.43
Condition factor ± SD	0.587**	0.649	0.547	0.652	0.545	0.485
(range)	±0.110	±0.047	±0.034	±0.117	±0.093	±0.050
	0.398–1.297	0.547–0.730	0.477–0.607	0.491–1.297	0.427–0.860	0.398–0.603
Prevalence (%)	18.6*	90.0	50.0	3.3	0	0
CI 95%	12.9–25.5	68.3–98.8	27.2–72.8	0.4–11.3	0.0–11.6	0.0–11.6
Mean intensity	4.67	6.61	1.70	1.50	0	0
(range)	(1–24)	(1–24)	(1–4)	(1–2)		
CI 95%	3.13–7.43	4.33–10.67	1.20–2.40	1.00–1.50	na	na
Mean abundance	0.87	5.95	0.85	0.05	0	0
CI 95%	0.51–1.50	3.75–9.60	0.45–1.40	0.00–0.15	Uncertain	Uncertain

Length in cm, weight in g. SD: standard deviation. Prevalence (P) = 100·N/F, mean intensity (MI) = A/N, mean abundance (MA) = A/F; where F is the total number of fish, N is the number of infected fish, and A is the number of larvae. CI: confidence interval. na: not applicable. \*\*Fish length, weight and condition factor between areas are significantly different when analysed by ANOVA ( $p < 0.0001$ ). \*The prevalence of all areas shows significant differences with Fisher's exact test ( $p < 0.0001$ ). <sup>1</sup> P, MI and MA parameters in the Bay of Biscay are significant when compared to the other areas ( $p < 0.05$ ). <sup>2</sup> P, MI and MA parameters in Portugal are significant when compared to the other areas ( $p < 0.05$ ), except for the MI in the Gulf of Cádiz. <sup>3</sup> P, MI and MA of the Gulf of Cádiz are not significant when compared with the two sampled areas of the Mediterranean Sea (Alborán Sea and Balearic Sea).



**Fig. 3.** Prevalence of *Anisakis* spp. L3 infection as a function of fish length. The sampled anchovies were grouped into 1 cm length classes. The number of fish per cm (or group) is 12, 29, 38, 35, 18, 15 and 14 anchovies, respectively from the lowest to the highest length class.

the ratio between these two species by location in/on the host, it is observed that, while the proportions in viscera and on the surface are similar, the proportion of *A. simplex* s.s. in muscle is 1.7 and 1.4 times higher in Bay of Biscay and Portugal, respectively, than that of *A. pegreffii* (Table 3).

Furthermore, 11 L3 of *H. aduncum* (10 from Ondarroa and 1 from the Gulf of Cadiz), identified morphologically and subjected to genetic identification, confirmed the specific identification as *H. aduncum*.

### 3.3. Molecular epidemiological parameters

Of the total number of anchovies sampled (161), only 30 ( $P = 18.6\%$ ) were parasitized by L3 of *Anisakis* spp.: 22 fish ( $P = 13.7\%$ ) by *A. simplex* s.s. (10 anchovies with larvae in muscle,  $P = 6.2\%$ ), 16 fish ( $P = 9.9\%$ ) by *A. pegreffii* (in muscle: 3 fish,  $P = 1.9\%$ ) and 6 fish ( $P = 3.7\%$ ) by hybrid genotype (in muscle: one fish,  $P = 0.6\%$ ). In both Bay of Biscay and Portugal, all 3 *Anisakis* genotypes were identified while in Gulf of Cádiz only *A. pegreffii* larvae were detected. In Bay of Biscay, the

prevalence in muscle was 40% with a mean intensity of 2.13; while in Portugal it was 20% and 1, respectively. The prevalence was 40% for *A. simplex* s.s., 5% for *A. pegreffii* and 5% for hybrid genotype in Bay of Biscay and 10% for both *A. simplex* s.s. and *A. pegreffii* in Portugal. However, it should be noted that 9 ( $P = 45\%$ ) of the anchovies had larvae on the surface (17 L3 *A. simplex* s.s. in 8 anchovies and 2 L3 *A. pegreffii* in two fish), all in Bay of Biscay. Data for each genotype are given in Table 4.

### 3.4. Analysis of risk factors of anchovy infection by *Anisakis*

In order to determine which variables could act as risk factors for *Anisakis* infection of fish in the three areas where infection was found, statistical analyses were carried out and showed that prevalence and intensity of infection depended on the length of the fish ( $p < 0.001$ ) and the area of origin ( $p < 0.001$ ). Post hoc analyses revealed that prevalence was higher in Bay of Biscay than in Portugal ( $p < 0.01$ ) and Gulf of Cádiz ( $p = 0.03$ ; Table 1), with no differences found between the latter



**Table 2**

Variation of the host and epidemiological parameters of *Anisakis* infection in anchovies according to length classes.

Parameters	Length (cm)		
	9.1–12.0	12.1–14.0	14.1–17.0
N° of fish	62	57	42
Length $\pm$ SD <sup>#</sup> (age)	11.2 $\pm$ 0.6 (1.0 y)	13.0 $\pm$ 0.5 (1.3 y)	15.3 $\pm$ 0.8 (1.8 y)
Weight $\pm$ SD <sup>#</sup>	8.65 $\pm$ 1.37	12.36 $\pm$ 3.31	20.81 $\pm$ 3.20
CF $\pm$ SD <sup>‡</sup>	0.615 $\pm$ 0.114	0.561 $\pm$ 0.121	0.582 $\pm$ 0.078
Prevalence (%)	0	5.3	64.3**
CI 95%	0–5.8	1.1–14.6	48.0–78.5
Mean intensity	0	1.00	5.04*
(range)		(1)	(1–24)
CI 95%	na	Uncertain	3.37–8.04
Mean abundance	0	0.05	3.24*
CI 95%	Uncertain	0.00–0.11	2.05–5.36

Weight in g, length in cm. CF: Fulton's condition factor. SD: standard deviation. na: not applicable. <sup>#</sup>When comparing length and weight between pairs of length classes, the differences are highly significant ( $p < 0.0001$ ). <sup>‡</sup>Differences between CF were only significant between the two smaller length classes ( $p < 0.05$ ). There were no parasitized anchovies  $<13.5$  cm ( $<1.37$  years); 3 anchovies parasitized  $<14.1$  ( $<1.50$  years); and 27  $> 14.1$  cm ( $>1.50$  years). When comparing the prevalence of the three length classes, a significance of  $p < 0.0001$  is observed. Likewise, there are significant differences between the epidemiological parameters (P, MI and MA) of the 14.1–17.0 cm class with the other length classes: \* $p < 0.05$ ; \*\* $p < 0.0001$ .

**Table 3**

Ratio between *A. simplex* s.s. and *A. pegreffii* in infected anchovies according to survey area and location in/on the host.\*

Area	Total	Viscera	Muscle	Surface
Bay of Biscay	9.3:1 (102/11)	8.8:1 (70/8)	15:1 (15/1)	8.5:1 (17/2)
Portugal	0.7:1 (8/11)	0.7:1 (6/9)	1:1 (2/2)	ND
Gulf of Cádiz	0:1 (0/3)	0:1 (0/3)	ND	ND

\* Results presented as ratio *A. simplex* s.s.:*A. pegreffii* larvae (number of L3 *A. simplex* s.s./number of L3 *A. pegreffii*). The hybrid genotype larvae were excluded. ND: not detected.

two ( $p = 0.24$ ). Similarly, Bay of Biscay anchovies exhibited higher intensity of infection than those from Portugal ( $p < 0.01$ ). However, no statistical differences were found between Gulf of Cádiz and Bay of Biscay ( $p = 0.52$ ), nor with Portugal ( $p = 0.09$ ), probably due to the low number of infected individuals in the first area ( $n = 2$ ).

As prevalence ( $p < <0.001$ ), intensity ( $p < <0.001$ ) and fish length ( $p < 0.002$ ) were found to vary between areas, these were analysed independently for prevalence and intensity. They were found to be

**Table 4**

Epidemiological infection parameters of anchovies by species of *Anisakis* genetically identified.

<i>Anisakis</i> genotype	Parameters	Bay of Biscay				Portugal			Gulf of Cádiz		
		Fish	Muscle	Surface	Viscera	Fish	Muscle	Viscera	Fish	Muscle	Viscera
<i>Anisakis</i> spp.	P (%)	90.0	40.0	45.0	80.0	50.0	20.0	35.0	3.3	0	3.3
	MI	6.61	2.13	2.11	5.19	1.70	1.00	1.86	1.5	0	1.5
	MA	5.95	0.89	1.00	4.15	0.85	0.20	0.85	0.05	0	0.05
<i>A. simplex</i> s.s.	P (%)	85.0	40.0	40.0	75.0	25.0	10.0	20.0	0	0	0
	MI	6.00	1.88	2.13	4.67	1.60	1.00	1.50	0	0	0
	MA	5.10	0.75	0.85	3.50	0.40	0.10	0.30	0	0	0
<i>A. pegreffii</i>	P (%)	40.0	5.0	10.0	30.0	30.0	10.0	20.0	3.3	0	3.3
	MI	1.38	1.00	1.00	1.33	1.33	1.00	1.50	1.5	0	1.5
	MA	0.55	0.05	0.10	0.40	0.40	0.10	0.30	0.05	0	0.05
Hybrids	P (%)	25.0	5.0	0	20.0	5.0	0	5.0	0	0	0
	MI	1.20	1.00	0	1.25	1.00	0	1.00	0	0	0
	MA	0.30	0.05	0	0.25	0.05	0	0.05	0	0	0

P = prevalence; MI = mean intensity; MA = mean abundance. Since all genotypes found in muscle have infective capacity on humans, the epidemiological data on larvae in muscle from the two areas where they are present (Bay of Biscay and Portugal) are compared, revealing non-significant differences. Considering that larvae on the surface could pose a risk to the consumer, we also compared the data for larvae found on the surface and in muscle in both areas (although no larvae were found on the surface in Portugal). This comparison shows a significant difference only for prevalence ( $p = 0.010$ ).

dependent on anchovy length in Bay of Biscay ( $p < 0.05$  and  $p < <0.001$ , respectively), only on prevalence in the Gulf of Cádiz ( $p < 0.001$ ) and only on intensity in Portugal ( $p < 0.003$ ). The coefficient of determination ( $R^2$ ) of the linear relationship between intensity and fish length was 0.511 in Bay of Biscay (Fig. 4) and 0.539 in Portugal. The analysis of the condition factor gave not-significant results.

#### 4. Discussion

Spain and Portugal are two of the countries with the highest fish consumption per capita/year in the European Union (see Eumofa data: <https://www.eumofa.eu/en/portugal> and <https://www.eumofa.eu/en/spain>). In 2015, >52,600 t of anchovies were caught by the fishing fleets of these two countries (Secretaría General de Pesca, 2017). It is therefore necessary to monitor the *Anisakis* infection in this fish of great commercial value in the Southern European countries, especially as some dishes, widely consumed in these countries, are prepared with raw anchovies. Although legislation obliges catering establishments to freeze this fish when it is to be served raw, this dish is traditionally prepared at home by the consumer and many either do not follow this recommendation or apply it incorrectly (see <https://youtu.be/10hleWIXsrc>, min. 4:45).

In this study, samples of fish landed at a variety of ports in the north, south, east and west of the Iberian Peninsula were collected in order to estimate the risk to consumers who do not follow the advice of health authorities and to individuals with *Anisakis* allergy, as many studies show an association between fish consumption and *Anisakis* sensitization (Figueiredo Jr et al., 2013; Heffler et al., 2016; Mazzucco et al., 2018; Valiñas et al., 2001).

The results obtained show significant differences between the prevalence of the different areas investigated: from 0% on the Mediterranean coast of the east and south of the Iberian Peninsula, to 90% on the Atlantic coast of Bay of Biscay in the north of the Peninsula, with increasing values as one moves clockwise along the coast from south to north (Fig. 1) both for prevalence and for mean intensity and mean abundance (Table 1).

These data are close to those previously published for the different areas, where available. Thus, although we have not found detailed prevalence data for *Anisakis* spp. in anchovies from Bay of Biscay, several authors have reported the presence of *Anisakis* or *Anisakis*-like larvae in samples from this area close to the Spanish coast (Dessier et al., 2016; Díez, 2001; ICES, 2002; Marigómez et al., 2006).

As we have found no previous reports of parasitism in anchovies landed at Portuguese fishing ports our prevalence data (50%) are the first to suggest that the Portuguese health authorities should consider implementing human anisakiasis prevention measures, particularly in

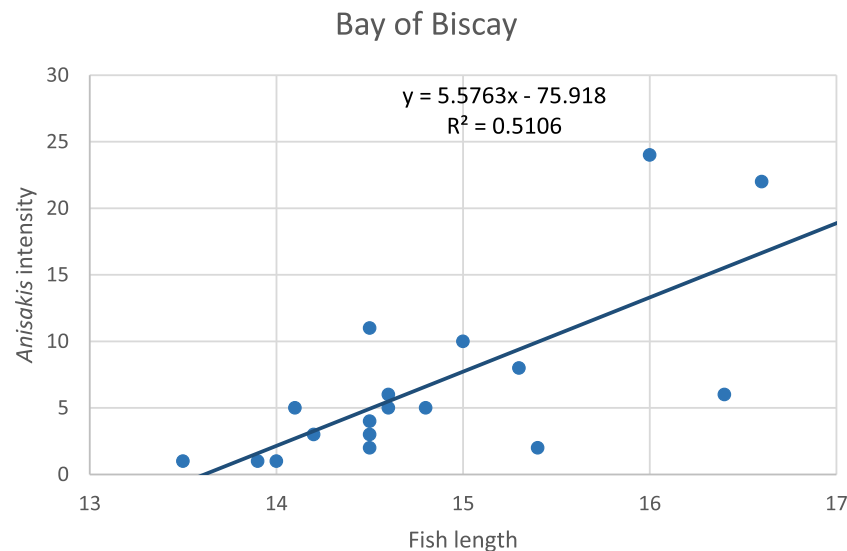


Fig. 4. Linear relationship between length of parasitized anchovies from Bay of Biscay and intensity of infection by *Anisakis* larvae.

view of recent studies showing an increasing tendency in the preparation of raw or undercooked fish dishes among the Portuguese population (Golden et al., 2022). Rello Yubero (2003) analysed 19 anchovies landed in Faro (Gulf of Cadiz, Southern Portugal) with an *Anisakis* prevalence of 5.3%. In the Gulf of Cadiz we found a prevalence of 3.3%, lower than the 13.1% previously reported (Rello et al., 2009). This highlights not only differences in epidemiological parameters between areas, but also differences over time within the same area (Díez et al., 2022), perhaps related to oceanographic and anthropogenic factors, such as pollution, overfishing (Cosín et al., 2015), global warming and associated climate change (Klimpel and Palm, 2011; Marcogliese, 2008).

Previously published data for anchovies from the Spanish Mediterranean coast report a prevalence between 0 and 2% consistent with the data in Table 1 (Cuéllar et al., 1991; Gutiérrez-Galindo et al., 2010; Rello et al., 2009; Rodríguez-Romeu et al., 2022; Valero et al., 2004).

Rello et al. (2009) reported significant differences in the prevalence of *Anisakis* in anchovies from the northern coast of the western Mediterranean Sea, progressively decreasing along the coast from the Ligurian Sea to the Alboran Sea, and increasing in the adjacent Gulf of Cadiz, in the NE Atlantic. Mattiucci et al. (2015) suggested that there may be regional differences between populations of anchovies and other fish in the Mediterranean Sea that even partially justify these differences. In this sense, the existence of several populations of anchovies has been suggested, supported by genetic studies (Zarraonaindia et al., 2012), which could lead to behavioural differences (migrations, habitats, diets, type of growth, ...). This could explain, at least in part, the variations observed in the present study, which shows a significant increase in the prevalence of *Anisakis* in anchovies when following the Atlantic coast of the Iberian Peninsula from south to north (Table 1). Moreover, the differences between studies may also be due to other biotic factors (abundance of parasite hosts, abundance of prey for these hosts, host life cycle, host age, nutrient abundance, ...) and abiotic factors (oceanographic conditions, water temperature and salinity, marine currents, upwelling phenomena, ...), including anthropogenic factors (Rodríguez-Romeu et al., 2022), that may change over the seasons and in different ways in each maritime area. For example, the variations in CF between areas (Table 1) have been positively correlated to, among other factors, the availability of food (Giráldez and Abad, 1995), which will undoubtedly vary between the different catch areas. In any case, caution should be exercised in view of the lack of studies on the influence of these factors on fish infection.

Furthermore, although the relationship between length and age may vary between different areas, we can calculate the approximate age of

anchovies from their length (Bellido et al., 2000), noting that anchovies <1.5 years (<14.1 cm) are not usually infected by *Anisakis* (Fig. 3 and Table 2). Considering that the diet of anchovies does not vary appreciably during their lifetime (Palomera et al., 2007), they may become infected from that age/size onwards as a consequence of the increased probability of ingestion of potential *Anisakis* hosts (either in numbers of prey or size) as the anchovy grows (Rello et al., 2009) (see Figs. 3, 4 and Table 2). This increase in *Anisakis* prevalence and/or intensity associated with fish size/age has been previously described for other host species (Brattey and Bishop, 1992; Buzo-Domínguez et al., 2021; Molina-Fernández et al., 2018, 2015; Morales-Yuste et al., 2022; Ruiz-Valero et al., 1992), although in other surveys largest fish are not the most infected (Bussmann and Ehrlich, 1979; Davey, 1972; Rello et al., 2008; Ruiz-Valero et al., 1992).

The presence of *Anisakis* on the surface of some anchovies has been related to their ability to migrate once the fish dies (Rello et al., 2009). Cipriani et al. (2016) show that the higher the storage temperature and the longer the time since capture, the greater the migration when anchovies are kept at temperatures >2 °C. In our survey, the appearance of larvae on the surface of the fish shows that transport conditions to fish outlets should be reviewed to ensure that the temperature does not exceed 2 °C. This migration has been observed in other fish species, but the data are controversial, especially in larger species (Cattan and Carvajal, 1984; Cipriani et al., 2015; Khalil, 1969; Smith and Wootten, 1975). In any case, it is recognised that L3 larvae of *A. simplex* s.s. have a greater capacity to penetrate the musculature than those of *A. pegreffii* (Cipriani et al., 2015; Debenedetti et al., 2019; Quiazon et al., 2011; Suzuki et al., 2010). In the present study, we have calculated the ratio *A. simplex* s.s.:*A. pegreffii* for catching area and for host location (viscera, muscle, surface), observing that the ratio in viscera and surface is similar although in muscle it increases in favour of a greater presence of *A. simplex* s.s., ratifying the studies that suggest a greater muscle penetration capacity of this species compared to *A. pegreffii* (Table 3; Cipriani et al., 2016; Suzuki et al., 2010). However, this muscle penetration capacity seems to vary between studies as Cipriani et al. (2016) reported that only 4% of the larvae recovered from *A. pegreffii* were in muscle, while in this study it varies between 9.1% in Bay of Biscay and 18.2% in Portugal, perhaps due to the time elapsed since the capture of the anchovies and the holding temperature during transport and distribution in the market, without ruling out other factors inherent to each sub-population of anchovies according to their origin (Zarraonaindia et al., 2012) or even intraspecific variability of the parasite. This also occurs in *A. simplex* s.s. (14.7% and 25.0% of L3 in Bay of Biscay and Portugal,

respectively, in muscle) but with higher percentages than in *A. pegreffii*, corroborating the greater penetration activity of the former (Table 3). This penetration capacity must be related to a histolytic activity of the larvae, probably due to proteases (Malagón et al., 2013). In this sense, a significantly higher proteolytic activity has been reported in *A. simplex* s. s., especially of serine proteases and cathepsin L, both frequently involved in tissue invasion (Molina-Fernández et al., 2019; Torralbo-Ramírez et al., 2019). On the other hand, the similar distribution between the two *Anisakis* species in viscera and surface, may mean that most of the larvae that appear on the surface come from the viscera and will have either emerged through natural orifices or as a result of damage to the muscle during fishing and transport of the anchovy, hence the proportion of species in viscera and surface is similar and, in turn, different from the muscle, suggesting that most of the larvae migrating to the surface must have come from the visceral package, underlining the importance of removing fish viscera immediately after capture for those fish species where this is possible.

The presence of L3 of *H. aduncum* in anchovies caught in the waters of the Iberian Peninsula is known but varies with the geographical area. Rello et al. (2009) reported prevalence of 37.6% in the NE of the Peninsula (Mediterranean coast; see Fig. 1) and much lower in the south (2.8% in the Mediterranean coast and 4.3% in the Atlantic coast), values close to those obtained in this study for the Gulf of Cadiz ( $P = 4.9\%$ ). Similar values were obtained for the Portuguese coast ( $P = 5.0\%$ ). The high prevalence ( $P = 95\%$ ) obtained in anchovies from the Bay of Biscay is in agreement with the report of Dessier et al. (2016) with values close to 100%. The number of the parasite hosts (mostly small crustaceans and fish) and suitable hydrographic conditions are key factors to explain, at least in part, these prevalence (Adroher-Auroux and Benítez-Rodríguez, 2021; Adroher et al., 1996; Klimpel and Rückert, 2005; Rello et al., 2009). A high prevalence, independently of *Anisakis* infection, represents an aesthetic problem that could be alleviated with fish evisceration since most of the larvae of this nematode are found in the visceral package. It should not be forgotten that it has not yet been clarified whether this parasite could infect or cause allergies in humans (Cavallero et al., 2020; Fernández-Caldas et al., 1998; Valero et al., 2003; Yagi et al., 1996).

It is known that the coast of the Iberian Peninsula (Fig. 1), at least from the Alboran Sea to the Bay of Biscay, is an area of sympatry between *A. simplex* s. s. and *A. pegreffii*, but the distribution varies clockwise from a higher proportion of *A. pegreffii* on the Alboran Sea coast to a higher proportion of *A. simplex* s. s. in Bay of Biscay, as already mentioned by Abaunza et al. (2004). Our results (Table 4) coincide with these observations since all the *Anisakis* collected from the south coast of the Iberian Peninsula have been identified as *A. pegreffii*, while on the west coast (Portugal) the frequency of each species was 50%, and in the north (Bay of Biscay) the ratio of *A. simplex* s. s. to *A. pegreffii* was approximately 9:1 (Table 3), data consistent with those obtained in blue whiting from Balearic Sea and from Bay of Biscay (Molina-Fernández et al., 2019; Torralbo-Ramírez et al., 2019). As both species of *Anisakis* are considered pathogenic (Romero et al., 2013) and capable of producing human anisakiasis (Adroher-Auroux and Benítez-Rodríguez, 2020; and references therein), it is not necessary to separate *Anisakis* prevalence data by species to understand the risk that the consumption of raw anchovies poses for the consumer who does not follow the anisakiasis prevention regulations, in addition to the risk for patients allergic to *Anisakis*.

## 5. Conclusions

New, larger, interdisciplinary studies that could determine the more precise effect of biotic and abiotic factors, such as those mentioned above, on the infection of fish by *Anisakis* spp. are required in order to better address the problem for the fishing industry and consumers caused by these parasites.

Authorities should regulate and monitor compliance with fish

processing and distribution regulations, especially to fishmarkets far from ports of landing, as the longer the time from catching the fish, the greater the likelihood of larval migration. In this regard, measures involving evisceration of fish at source should be prioritised, where possible.

Finally, health authorities should take note of these studies, be vigilant and remind the fish consuming population of the need to process the fish by proper thermic treatment to inactivate the parasite ( $-20\text{ }^{\circ}\text{C} > 24\text{ h}$  or  $> 60\text{ }^{\circ}\text{C} > 1\text{ min}$ ) (EU, 2011).

## Ethics statement

No ethical review and approval was required for the fish survey, as the fish were collected from fish markets.

## Author contributions

Conceptualization, F.J.A., M.M.-Y. and R.B.; methodology, M.M.-Y., S.B.-D. and A.M.D.-H.; data analysis, F.J.A., R.B. and M.M.-Y.; parasitological examination, S.B.-D. and A.M.D.-H.; molecular analysis, M.M.-Y., S.B.-D. and A.M.D.-H.; writing—original draft preparation, F.J.A., M. M.-Y. and R.B.; writing—review and editing, F.J.A., M.M.-Y. and R.B. All authors have read and agreed to the published version of the manuscript.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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