Title: Fishing area and fish size as risk factors of *Anisakis* infection in sardines (*Sardina pilchardus*) from Iberian waters, southwestern Europe.

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ABSTRACT

The sardine (*Sardina pilchardus*) is a fish commonly consumed and appreciated in many countries, although they are more likely to be eaten fresh in western Mediterranean countries such as Spain, Portugal, France or Italy. A molecular epidemiological survey of sardines from 5 fishing areas of the Spanish Mediterranean (Málaga, southern Spain) and Atlantic coasts (southern: Cádiz and Isla Cristina; northern: A Coruña and Ondarroa) was carried out to determine the presence of *Anisakis* spp. larvae. The highest prevalence of these larvae was observed in fish from A Coruña (28.3%), followed by Ondarroa (5%) and Cádiz (2.5%). No *Anisakis* larvae were found in fish from Málaga and Isla Cristina. Three *Anisakis* genotypes were identified: *A. simplex sensu stricto, A. pegreffii* and a hybrid genotype between these two species. *A. pegreffii* was the most prevalent species in A Coruña (71 % of larvae). Only three *Anisakis* larvae (9% collected larvae) were located in the musculature of sardines: two were identified as *A. pegreffii* while the other was a hybrid genotype. Sardine infection was associated with fishing area and fish length/weight (length and weight were strongly correlated; Pearson's correlation 0.82; p<0.001). Risk factor multivariate analysis showed that the risk of infection increases 1.6 times for every additional cm in the length of the sardines from the same fishing area. Comparison of fish of equal length showed that in sardines from A Coruña the risk of parasitisation is 11.5 times higher than in those from other fishing areas. Although the risk of infection by *Anisakis* through consumption of sardines is generally low due to the low epidemiological parameter values (prevalence 10%, mean intensity 1.7 (range 1-5) and mean abundance 0.17), as larger fish are more heavily parasitized, there is an increased risk of infection by *Anisakis* through consumption of large sardines which are raw or have undergone insufficient heat treatment (undercooked,smoked, marinated, salted, pickled,…).

Keywords: *Sardina pilchardus*, anisakiasis, *Anisakis simplex s.l.*, hybrids, epidemiology, infection risk factors.

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1. Introduction

The sardine (*Sardina pilchardus*) is a littoral fish which feeds mainly on planktonic crustaceans, appendicularians, diatoms and other organisms (Costalago and Palomera, 2014). This fish is marketed fresh, frozen or canned. It is also consumed dried or salted and smoked or marinated and can be pan-fried, broiled and microwaved. Sardines can harbour parasites such as *Anisakis*, which are transmitted to humans.

Anisakis spp. are nematodes which can parasitize a wide range of marine animals. The third larval stage (L3) of this parasite is the etiological agent of human anisakiasis, a disease that causes gastric and intestinal illness and/or allergic reactions. The larvae of *Anisakis*, dead or alive, are also considered to cause food allergy, although this is currently under discussion (Audicana and Kennedy, 2008; Daschner et al., 2012). Reports of cases of anisakiasis are increasing globally. The majority of cases have been reported in Japan, where consumption of raw fish is extremely common.

The life cycle of *Anisakis* is complex and involves a large number of host species. Larvae of *Anisakis* have been reported in numerous invertebrate hosts, mainly crustaceans, which can act as intermediate hosts. L3 of this parasite have been found in a wide range of fish and cephalopods, which are intermediate/paratenic hosts. *Anisakis* parasitization has been reported in more than 200 fish and 25 cephalopod species (Abollo et al., 2001; Klimpel et al., 2004). Cetaceans (final hosts) harbour the adult stage of this nematode. Humans can become accidental hosts, by eating raw, marinated or undercooked fish containing the L3 of these parasites that have not been inactivated during preparatory procedures.

Anisakis type I larvae have been categorized into six species: *Anisakis simplex sensu stricto*, *A. pegreffii*, *A. berlandi*, *A. typica*, *A. ziphidarum* and *A. nascetti* (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009; Mattiucci et al., 2014). Although several species have been found to parasitize fish and cephalopods in Japan, human anisakiasis is caused almost exclusively by *A. simplex s.s.* larvae in this country (Arizono et al., 2012; Umehara et al., 2007). Eleven clinical cases attributed to *A. pegreffii* have been reported in Italy, where this species is the dominant in Italian waters whereas none due to *A. simplex s.s.* have been described to date in this area (D'Amelio et al., 1999; Fumarola et al., 2009; Mattiucci et al., 2012). In Spain, since 1991, when Arenal Vera et al. described the first case of anisakiasis, hundreds of new cases of *Anisakis* infection and *Anisakis* allergy have been described, although no cases have yet been diagnosed molecularly. Several studies conducted in Spain in healthy individuals have revealed a high seroprevalence (12-22%) to *Anisakis* related to the high consumption of fish from the Spanish population (Del Rey Moreno et al., 2006; Fernández de Corres et al., 2001; Puente et al., 2008). Among the cases reported, at least 4 of hypersensitivity to *Anisakis* have been linked to the consumption of either fresh or canned sardines (Audicana and Kennedy, 2008), and 3 of human anisakiasis to consumption of marinated sardines (Barros et al., 1992; López-Vélez et al., 1992). Two sibling species, *A. simplex s.s.* and *A. pegreffii*, are sympatric off the Atlantic coasts of the Iberian Peninsula and in the Alborán Sea (Martín-Sánchez et al. 2005; see Mattiucci and Nascetti, 2008 for

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references). Previous studies showed high parasitization in several species of fish by these two parasites (Abollo et al., 2001) but little or no parasitization in sardines from Spanish coasts and fishmarkets (see Fig. 1 and Table 1) (Abollo et al., 2001; De la Torre Molina et al., 2000; Gutiérrez-Galindo et al., 2010; Rello et al., 2008, Ruiz-Valero et al., 1992; Viu et al., 1996). However, Silva and Eiras (2003) recorded 28.1% prevalence of *Anisakis* sp. in 57 sardines from the west coast of Portugal, although the mean intensity was low. Romero et al. (2013), working with rats, defined the pathogenic potential of the *Anisakis* larva as its capacity to cause lesions, attach itself to the gastric or intestinal wall, or penetrate them to reach the abdominal cavity. The results obtained by these authors show that *A. simplex s.s.* is more pathogenic than *A. pegreffii*. Due to these differences in the pathogenic potential and the importance of the sardine in the cuisine and economy of the Western Mediterranean, it is useful to carry out a survey to identify the species infecting this host and the risk factors of *Anisakis* infection in sardines from Spanish waters. Fish consumption is high in Spain (Welch et al., 2002) with the fishing fleets of Spain and Portugal landing over 77,000 tons of sardines in 2013 only from the Atlantic Iberian waters, IXa and VIIIc ICES sub-areas(ICES, 2014).

2. Material and methods

2.1. Host and parasites

A total of 190 sardines (*Sardina pilchardus* Walbaum, 1792; family Clupeidae) from 5 Spanish ports on the Mediterranean (port of Málaga) and Atlantic coasts (Southern Spain: Cádiz and Isla Cristina; Northern Spain: A Coruña and Ondarroa) were surveyed between October 2011 and November 2012 (Fig. 1). After measuring the total length and weight, the fish were dissected to harvest the larvae. 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 an indicator of general fish health. The viscera and the muscle were each subjected to a pepsin digestion (pH 2), as described by Huss and Drewes (1989), at 37 ºC for 2 hours for the former and 6 hours for the later. All larvae morphologically identified (Berland, 1961; Petter and Maillard, 1988) as *Anisakis* L3 type I *sensu* Berland (1961), were individually preserved in Eppendorf tubes at -20 ºC for genetic identification studies.

2.2. Genetic identification of the parasites

For the genetic identification study of the larvae, polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) of the ribosomal fragment ITS1-5,8S-ITS2 was carried out. Realpure Kit was employed to extract the genomic DNA of every larva. PCR amplification primers NC5 (forward): 5' GTAGGTGAACCTGCGGAAGGATCATT 3', and NC2 (reverse) 5' TTAGTTTCTTTTCCTCCGCT 3', described by Zhu et al. (1998), were employed. Amplifications were carried out with the following programming: one cycle of 94 ºC for 5 min, 60 ºC for 60 s, 72 ºC for 90 s; 35 cycles of 94 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 60 s; and one final cycle of 94 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 5 min, and then cooled and kept at 4 ºC until use. The expected size of the amplified fragment was 1000 bp. PCR products were run on gels prior to digestion to verify the success of the amplification process. As controls, we used 2 specimens previously identified by this same technique as *A. pegreffii* and another 2 identified as *A. simplex s.s.* (Martín-Sánchez et al. 2005). RFLP was performed independently with two restriction enzymes, *TaqI* (5'...T↓CGA...3') and *HinfI* (5'...G↓ANT...3') Fast Digest (Thermo Sciencific) at 65ºC and 37ºC for 10 min, respectively, using a final enzyme concentration of 0.5 U/µl.. The results were visualized through electrophoresis in 3% agarose gel, which permitted the sibling species of *A. simplex* complex to be identified according to the band pattern. In *A. simplex s.s.* controls, digestion with *HinfI* enzyme produced two fragments of 620 and 250 bp as well as a weaker one of 100 bp; *TaqI* endonuclease provided three fragments: one of 430 bp, one of 400 bp and a weak one of 100 bp. The *A. pegreffii* controls presented a pattern of three bands of 370, 300 and 250 bp for *HinfI* enzyme and three of 400, 320 and 150 bp for *TaqI* enzyme. For hybrid individuals, PCR-RFLP band pattern with the two restriction enzymes, *HinfI* and *TaqI*, is the sum of the patterns generated for *A. simplex s.s.* and *A. pegreffii*.

2.3. Epidemiological parameters and statistical analysis.

The epidemiological parameters such as prevalence, mean abundance and mean intensity of *Anisakis* infection in sardines, were calculated as defined by Bush et al. (1997), i.e., "prevalence is the number of hosts infected with 1 or more individuals of a particular parasite species (or taxonomic group) divided by the number of hosts examined for that parasite species"; "mean intensity is the average intensity of a particular species of parasite among the infected members of a particular host species"; and "mean abundance is the total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined". Differences between prevalence values were evaluated by using the chi-square test or Fisher's exact test, with 95% confidence intervals being determined when possible while a bootstrap 2-sample t-test was used to compare mean intensities and mean abundances. These analyses were performed using free Quantitative Parasitology 3.0 computer software developed by Reiczigel and Rózsa (2005) to address the notoriously left-biased frequency distributions of parasites, based on the theoretical background published by Rózsa et al. (2000).

2.4. Analysis of risk factors of sardine infection.

For analysis of risks, the following variables of the sardines were studied as potential risk factors for infection by *Anisakis*: length, weight, sex, condition factor, Atlantic/Mediterranean origin, fishing area (port where landed) and catch month. A univariate model considering parasitization as the dependent variable, and the above factors as independent variables was developed. Next, a multivariate model selecting variables according to the statistical significance of their association with parasitization was developed. SPSS 20.0 was used for the data analysis.

3. Results

3.1. Host

The mean length±SD (standard deviation) of sardines was 20.9±1.4 cm (n=190). The mean weight±SD was 81.0±21.4 g (n=190) (Table 2). The relationship between weight and length shows a potential line with exponent around 3 ($W = 0.0065 \cdot L^{3.094}$; coefficient ± 0.0025 and exponent ± 0.128; $R^2 = 0.7577$), thus demonstrating a relationship generally accepted in the literature as cubic (Fig. 2; Fulton, 1904; see Nash et al., 2006). In this sense, total length and weight were strongly correlated (Pearson's correlation 0.82; p<0.001). The mean CF±SD per fish was 0.87±0.12 (n=190) and per fishing areas was 0.86±0.06 (n=5) (Table 2).

Nineteen of the 190 sardines analysed were infected (10%) and 33 *Anisakis* larvae found, all of which were identified morphologically as third larval stage (L3) type I *sensu* Berland (1961). The highest prevalence was found in fish from A Coruña (28.3%), followed by Ondarroa (5%) and Cádiz (2.5%). No parasitization was found in sardines from Málaga and Isla Cristina. Three larvae (9%) were isolated from the muscle of two fish, both from A Coruña. One of these sardines hosted four L3, two in viscera and two in musculature. The remaining larvae were found in the abdominal cavity, free or encapsulated on viscera of the hosts (91% of larvae). A mean intensity of 1.8 (range 1-5) and mean abundance of 0.5 were calculated in fish from A Coruña (Table 2). A relationship between intensity and fish length is depicted in Fig. 3. The mean intensity was 1.7 (range 1-5) and the mean abundance was 0.17 in all the fish examined in this survey.

3.2. Genetic identification of *Anisakis* larvae type I by PCR-RFLP

All thirty-three larvae isolated from sardines morphologically identified as *Anisakis* larva type I *sensu* Berland (1961) were further classified by genetic markers: 21% (7/33) were identified as *A. simplex s.s.*, 70% (23/33) as *A. pegreffii* and 9% (3/33) showed a hybrid PCR-RFLP band pattern with the two restriction enzymes used (TaqI and HinfI). Two of the three *Anisakis* larvae type I isolated from the muscle were identified as *A. pegreffii*; the other was identified as a hybrid genotype. The sole larva collected from Ondarroa was *A. simplex s.s.* and that from Cádiz was *A. pegreffii*.

3.3. Molecular epidemiological parameters.

The epidemiological parameters, following molecular diagnosis of the L3 of *Anisakis* spp. collected from sardines landed in A Coruña, are shown in Table 3. *A. pegreffii* was 3.5-fold more prevalent than *A. simplex s.s.* ($p < 0.02$) in an area considered sympatric for these species while the mean abundance of *A. pegreffii* was 3.7-fold that of *A. simplex s.s.* ($p = 0.05$). However, the mean intensities were similar ($p = 0.91$). Hybrids of these two species showed similar epidemiological parameters to *A. simplex s.s.* (Table 3).

3.4. Analysis of risk factors of sardine infection.

Total length, total weight and fishing area have shown their association with parasitization in both univariate and multivariate models. Lack of association has been found with catch month and Atlantic/Mediterranean origin. Sex and CF of the sardine show an association with parasitization in a univariate model but a lack of association in a multivariate model.

Univariate models: Using the port of Cádiz as the reference for fishing area, sardines from Málaga, Isla Cristina and Ondarroa have the same risk of parasitization as those from Cádiz (p=0.62). However, sardines from A Coruña have a risk of infection 15 times higher than those from Cádiz (p<0.01). Furthermore, statistical association between fish total length/weight and parasitization by *Anisakis* have been demonstrated in this analysis. The risk of infection is multiplied by 2.4 for every additional cm in the length of the sardine. Sardines over 22.5 cm have a risk of parasitization 14 times higher than those under 21.0 cm (p<0.01). The risk of infection increases 6.6% for every additional gram of fish weight. The risk of infection by *Anisakis* is 13 times higher in sardines over 110 g than in those under 90 g (p<0.01).

Multivariate models: Total length and weight were strongly correlated as indicated above. Consequently, one of them was excluded from the multivariate model, total length being selected to display this model. In fish of equal length, sardines from A Coruña have a risk of parasitization 11.5 times higher than those from Cádiz (OR= 11.5; p= 0.02). Within the same fishing area, the risk of parasitization is multiplied by 1.6 for every additional cm in fish length (OR=1.6; p<=0.05).

4. Discussion

As sardines are widely caught and consumed in Spain it is useful to know the prevalence of infection by *Anisakis* in this host and to estimate the risk of human anisakiasis due to the consumption of this fish (Table 1). At least three human anisakiasis cases and four *Anisakis* allergy cases associated with consumption of sardines have been described in Spain (Barros et al., 1992; López-Vélez et al., 1992; Audicana and Kennedy, 2008). No parasitization in sardines (*S. pilchardus*) from Spanish Mediterranean coasts has been found, in agreement with other authors who have also reported no parasitization by *Anisakis* in sardines from this area (Cuéllar et al., 1991; Gutiérrez-Galindo et al., 2010; Rello et al., 2008). Several authors have also reported absence of *Anisakis* infection in sardines from Spanish coasts (Abollo et al., 2001; De la Torre Molina, 2000; Pereira Bueno, 1992; Viu et al., 1996). Sardines of the Western Mediterranean area, when parasitized, generally show a low prevalence of less than 5% (Ruiz-Valero et al., 1992; see Table 1), except in Sardinian waters (Piras et al., 2014). However, NE Atlantic surveys show a generally higher prevalence, such as occurs in sardines from the coast of NW Portugal with 28.1% prevalence (Silva and Eiras, 2003) and 10.7% larvae found in the muscle. In our study, 28.3% prevalence was found in sardines from A Coruña, and 9.1% of the total number of isolated larvae were found in the muscle, similar data to those from Portuguese waters, probably due to the proximity between the surveyed areas from NW Iberian Peninsula [Porto (Portugal) and A Coruña (Spain), see Fig. 1]. *A. pegreffii* was the dominant species in this survey (70%). The only larvae to penetrate the fish muscle were 2 *A. pegreffii* and 1 hybrid of these two species, in agreement with the higher prevalence of *A. pegreffii* with respect to A. *simplex s.s.*. Differentiation between *A. simplex s.s.* and *A. pegreffii* using ribosomal DNA markers is based exclusively on the existence of two fixed differences (two C/T transitions) at positions 255 and 271 in the ITS-1 sequence, meaning that different restriction patterns are produced with *HinfI* and *TaqI* enzymes (Abollo et al., 2003; Ceballos-Mendiola et al., 2010). The restriction enzyme *Cfo* was not used in this study since it generates the same pattern for the two sibling species. The detection of the mix of genotypes of both species (hybrid genotypes) has been the cause of some controversy in terms of its interpretation. While some authors believe these mixed genotypes reflect hybridization, others adduce incomplete homogenization in a multiple-copy repeated DNA region (Martín-Sánchez et al., 2005; Hermida et al., 2012). *A. pegreffii* has a lower capacity to penetrate fish musculature (Suzuki et al., 2010; Quiazon et al., 2011) and rat gastrointestinal wall (Romero et al., 2013) compared to *A. simplex s.s.* Despite these differences, *A. pegreffii* is also capable of penetrating fish muscle and causing lesions in rats and human anisakiasis (Fumarola et al., 2009; Romero et al., 2013). The presence of *Anisakis* larvae in fish muscle poses a greater risk of infection for humans since this is the preferred part of the fish for consumption. These larvae were found in two sardines from A Coruña (1% of all surveyed fish; 3% of fish from A Coruña). Further north in the Atlantic, outside the boundary extension of *A. pegreffii* (Ceballos-Mendiola et al., 2010), Karl (2008) conducted another survey in sardines from southern Great British waters showing 50% prevalence, but only 0.1% larvae in muscle. These data suggest an increase in *Anisakis* parasitism of sardines with increasingly northern latitude in the NE Atlantic Ocean (see Table 2 with data from Isla Cristina and Cádiz). Sardine growth performance is generally lower in the Mediterranean and increases across the northeastern Atlantic from Northern Morocco to the English Channel (Silva et al., 2008). Our results show that the larger sardines were the most parasitized (Fig. 3; see analysis of risk factors in section 3.4) and that these came from the waters of NW Spain. This association between fish length/weight and *Anisakis* parasitization has long been known in other fish species (Abattouy et al., 2011; Adroher et al., 1996; Grabda, 1974). In addition, the *Anisakis* infection risk in sardines of equal length is 11.5 times higher in fish from A Coruña, i.e., the infection is associated with fishing area, as suggested previously by Rello et al. (2009) for anchovies. The size of the fish has been identified as a risk factor for *Anisakis* infection in other species such as horse mackerel (*Trachurus trachurus*) or mackerel (*Scomber japonicus*). Similarly to our results, no association was observed in the horse mackerel between *A. pegreffii* infection and Atlantic/Mediterranean catch area (Abattouy et al., 2014). In contrast, the risk of parasitization was reported to be more than three times higher in mackerel from Atlantic versus Mediterranean waters of the Moroccan coast (Abattouy et al., 2011). We also found no differences in *Anisakis* presence between the sexes of sardines, consistent with results obtained in other species of fish (Abattouy et al., 2011, 2014). Fish CF was not related to parasitization in the multivariate model analysis, suggesting that the higher prevalence of *Anisakis* in sardines from A Coruña (highest CF, Table 2) is related more to the lifespan of the sardines and to the fishing area than to the condition factor, an index of apparent health of fish. In this way, most marine fish ecologists currently consider that the dietary habits of a fish species may depend upon both the availability of prey and the anatomy of the fish

(Costalago and Palomera, 2014 for references).

The scarce *Anisakis* parasitization of smaller sardines could be explained by the fact that they feed mainly on copepods and phytoplankton (Palomera et al., 2007). The infection by *Anisakis* is suspected to occur when fish become bigger and euphausiids, known intermediate hosts of *Anisakis*, and other components of the plankton are incorporated into their diet (Cunha et al. 2005; Palomera et al. 2007 for references). Koie (2001) showed that the copepods can be directly infected by ingesting L3 of *Anisakis*, at least experimentally, but these larvae were not developed. The euphausiids were infected by eating these infected copepods, and thus the sardines could not have been infected by *Anisakis* via copepods but rather by eating euphausiids (in the case of large sardines), in which the L3 are developed (Fig. 3). Conversely, Klimpel et al. (2004) showed that, in Norwegian waters, the *Anisakis* lifecycle is sustained using only large carnivorous copepods as first intermediate hosts and planktivorous small fish as second intermediate hosts, without utilizing euphausiids. These authors suggested that *Anisakis* has a great ability to adapt its lifecycle to the autochthonous marine hosts. On the other hand, the higher prevalence and intensity in the largest fish could be also explained by the accumulation of parasites over the life of the fish (Bussmann and Ehrich, 1979; Valero et al., 2000) since these larvae can survive up to 3 years in fish (Smith, 1984). In agreement with Rello et al. (2008), we also suggest that a lower frequency of euphausiids in the Iberian Mediterranean pelagic waters versus Iberian Atlantic waters and the higher presence of cetaceans in the latter, facilitate the maintenance of the *Anisakis* lifecycle in the Atlantic waters of the Iberian Peninsula (Aguilar Vila et al., 1997; Anon. 2012; Furnestin, 1968; Papetti et al., 2005; Raga and Pantoja, 2004). The estimated population of cetaceans in Atlantic Iberian Peninsula waters is about 30,000 dolphins and porpoises (Santos et al., 2014), plus the migrating cetaceans. Sardine is the main prey (in terms of reconstructed prey biomass) of the common dolphin in Portugal and second in importance (after blue whiting, *Micromesistius poutassou*) in the dolphins of Galician and Portuguese waters (Santos et al., 2014 for references). The sardine could thus act as an intermediate/paratenic host in the *Anisakis* lifecycle in these Atlantic waters, although blue whiting are frequently parasitized by *Anisakis* and could also act as a source of cetacean infection (Ruiz-Valero et al., 1992).

A. simplex s.s. is the prevalent *Anisakis* species in the Atlantic Ocean. This species is widely distributed throughout the eastern Atlantic Ocean, its southern limits being the waters of the Strait of Gibraltar (Mattiucci and Nascetti, 2008). On the other hand, according to Mattiucci and Nascetti (2008), *A. pegreffii* is the dominant species in the Mediterranean Sea (Romero et al., 2013, observed that the likelihood of finding *A. pegreffii* L3 larvae in blue whiting from Spanish Mediterranean waters is six times higher than in those from Spanish Atlantic waters) although it is also present in Atlantic waters, its northern limits being the North Spanish coast. Therefore, a sympatric area between *A. simplex s.s.* and *A. pegreffii* has been identified along the Spanish and Portuguese Atlantic coasts and in the Alborán Sea (Martín-Sánchez et al. 2005; Mattiucci et al., 2008 for references). Thus, it is not uncommon to find hybrids of these two species (Abbatouy et al., 2011 and 2014; Abollo et al., 2003; Ceballos-Mendiola et al., 2010; Hermida et al., 2012; Martín-Sánchez et al. 2005; and others), although Mattiucci et al. (1997) suggested that paratenic hosts of *A. simplex s.s.*

were mainly benthic and demersal whereas those of *A. pegreffii* were mainly pelagic. However, as in other fish species, our results show both parasites and their hybrids in a pelagic host albeit with a 3.3-fold greater number of larvae of *A. pegreffii* (Table 3), since 71% of larvae isolated from sardines from A Coruña were identified as *A. pegreffii*.

In summary, we have shown that fishing area is a risk factor of *Anisakis* infection in sardines from Iberian waters, as suggested previously by Rello et al. (2009) for anchovies from the western Mediterranean and the Gulf of Cádiz. Our results also show greater prevalence and intensity with greater sardine size demonstrating that fish size is other risk factor. Although the low number of larvae found in the muscle tissue of the fish represents a lower likelihood of human infection or allergy to *Anisakis* —the meat is the preferred part of the fish for consumption—, cases of human anisakiasis through consumption of marinated sardines have been reported in Spain. Clearly, the risk of infection is lower if small sardines or those from fishing areas with low prevalence of *Anisakis* infection are consumed. For example, a sardine of 25.5 cm caught in the waters of A Coruña is 550 times more likely to be parasitized by *Anisakis* than one of 17.6 cm captured in any of the other sample areas. Likewise, a sardine of lower weight also represents a lower infection risk. Thus, one sardine of 112 g presents a risk of infection 18 times higher than two sardines of 56 g. However, the most effective method of preventing human anisakiasis through consumption of sardine or any other fish is to follow public health guidelines; that is, to eat only fish which has undergone a suitable freezing (more than 24 hours at -20 ºC for whole mass) or cooking process (attaining an internal temperature of more than 60 ºC for at least 10 minutes) (EEC, 1991).

However, it is still under discussion whether or not these measures prevent allergy to *Anisakis*, as a food allergy problem (Audicana and Kennedy, 2008; Daschner et al., 2012). Therefore, until this issue is resolved, it may be advisable for people with *Anisakis* allergy to consume smaller fish, which will also reduce the risk of anisakiasis. In addition, knowledge of fishing areas with lower parasite prevalence may be of interest to fishing fleets, which will be able to offer a fish of higher sanitary quality and will also suffer fewer economic losses caused by confiscation of infected fish by the health authorities. The relationship between these studies and the culinary habits of the people of a country or region could enable health authorities to be prepared for the possible incidence of this infection / allergy in the population.

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Table 1.- *Anisakis* parasitization in sardines *Sardina pilchardus*. Published surveys.

References	Sardines analyzed	Origin	Sardines parasitized (Prevalence of *Anisakis* spp.)	Mean intensity (range)
Petter, 1969	400	NE Atlantic Ocean: Le Croisic, Region Nantaise, (Western France)	0	-
Carvalho-Varela & Cunha-Ferreira, 1984	310	Portuguese waters	0	-
Petter & Maillard, 1988	?	Castiglione, Algeria	Yes	?
Huang, 1988	22	Paris-Rungis fishmarket (Northern France)	1 (4.5%)	1 (1)
Cuéllar et al., 1991	?	Castelló waters (Eastern Spain)	0	-
Ruiz-Valero et al., 1992	310	Granada fishmarket (Southern Spain)	3 (0.9%)	1.3 (1-2)
Pereira Bueno, 1992	44	Bilbao fishmarket (Northern Spain)	0	-
Sanmartín et al., 1994	20	Galician coasts (NW Spain)	2 (10%)	1 (1)
Viu et al., 1996	204	Zaragoza fishmarket (NE Spain)	0	-
De la Torre Molina et al., 2000	294	Northern area of Córdoba province fishmarkets (Southern Spain)	0	-
Abollo et al., 2001	50	Galician coasts (NW Spain)	0	-
Silva & Eiras, 2003	57	West Portuguese coasts-Porto waters	16 (28.1%)#	
Fioravanti et al., 2006	1323	Adriatic Sea	2 (0.1%)	1 (1)
Karl, 2008	100	South of Great Britain	50 (50%)#	6.3 (?)
Rello et al., 2008	350	-Mediterranean Spanish coasts: Roses, Barcelona, Tarragona, Castelló, Almería, Adra, Málaga. -Atlantic south Spanish coasts: Barbate, Cádiz.	0	-
Kijewska et al., 2009	11	Northwest African shelf between Morocco and Mauritania	0	-
Gutiérrez-Galindo et al., 2010	160	Tarragona waters (East Spain)	0	-
Angelucci et al., 2011	5	Sardinian waters	1 (20.0%)	1 (1)
Chaligiannis et al., 2012	36	Southern Aegean Sea	2 (5.5%)	1 (1)
Fioravanti et al., 2012	2636:	Mediterranean Sea:	5 (0.2%)	~1 (?)
	1591	NW Adriatic	3 (0.2%)	
	1045	Ligurian coasts	2 (0.2%)	
Cavallero & D'Amelio, 2012	93	Fishmarkets in central Italy	1 (1.1%)	3 (3)
Mladineo et al., 2014	120	Croatian coast, Adriatic Sea	4 (3.3%)	1.25 (1-5)
Piras et al., 2014	252	Gulf of Asinara, Northern Sardinia, Mediterranean Sea	33 (13.1%)#	1.2 (1-3)
Serracca et al., 2014	750	Ligurian Sea coast (NW Italia)	0	-
This report	190:	Spanish coasts:	19 (10%)	1.74 (1-5)
	60	-A Coruña (NW)	17 (28.3%)#	1.82 (1-5)
	20	-Ondarroa (N)	1 (5%)	1 (1)
	40	-Cádiz (S)	1 (2.5%)	1 (1)
	30	-Isla Cristina (S)	0	-
	40	-Málaga (S)	0	-

Presence of larvae in muscle of sardines is described. Prevalence: 2.8% (Piras et al., 2014); 3.3% A Coruña and total this survey 1.1%; 10.7% (Silva and Eiras, 2003); 0.1% of all larvae in flesh, prevalence ~1% (Karl, 2008).

Table 2.- Epidemiological parameters of sardines parasitized by *Anisakis* spp. from the surveyed areas of Iberian waters.

	West Mediterranean Sea	North-East Atlantic Ocean			
		South Spain		NW Spain	North Spain
	Málaga	Cádiz	Isla Cristina	A Coruña	Ondarroa
No. fish	40	40	30	60	20
Mean weight ± SD	68.4 ± 17.7	76.1 ± 11.6	74.8 ± 7.4	99.6 ± 17.7	69.9 ± 8.9
(range)	(45.6-101.3)	(55.8-98.9)	(58.5-92.4)	(63.3-198.3)	(56.1-94.7)
Mean length ± SD	19.7 ± 1.7	21.3 ± 0.8	20.4 ± 0.8	21.9 ± 1.2	20.3 ± 0.9
(range)	(17.6-22.3)	(19.4-23.0)	(19.5-22.0)	(19.8-25.5)	(18.9-22.1)
Condition factor ± SD	0.87 ± 0.07	0.78 ± 0.07	0.85 ± 0.08	0.95 ± 0.15	0.83 ± 0.04
(range)	(0.66-1.04)	(0.59-0.94)	(0.70-1.02)	(0.77-1.91)	(0.76-0.88)
Prevalence (%)	0	2.5	0	28.3	5
Mean Intensity	-	1	-	1.82	1
Mean Abundance	-	0.025	-	0.52	0.05

Weight in g; Length in cm. SD=standard deviation. Prevalence=100·*N/F*, mean intensity=*A/N*, mean abundance=*A/F*; where *F* is the total number of fish, *N* is the number of infected fish, and *A* is the number of larvae.

Table 3.-Taxa of *Anisakis* type I larvae identified by genetic markers from sardines from the Atlantic waters of A Coruña.

	Prevalence (%) 95% CI[a]	Mean Intensity (range) 95% CI	Mean Abundance 95% CI
Anisakis spp.	28.3 18.2-40.8	1.82 (1-5) 1.29-2.65	0.52 0.30-0.87
Anisakis pegreffii[b]	23.3* 13.9-35.7	1.57[ns] (1-5) 1.14-2.43	0.37* 0.18-0.63
Anisakis simplex s.s.	6.7 2.3-16.4	1.50 (1-3) 1.00-2.00	0.10 0.02-0.25
Anisakis hybrid	5.0 1.38-13.91	1.00 (1)[uc]	0.05 0.00-0.10

Prevalence=100·*N/F*, mean intensity=*A/N*, mean abundance=*A/F*; where *F* is the total number of fish, *N* is the number of infected fish, and *A* is the number of parasites.

[a] CI: confidence interval.

[uc] 95% confidence limits are uncertain.

[b] Statistical analysis to compare epidemiological parameters by *A. pegreffii* versus *A. simplex s.s.* and hybrids was statistically significant (*) for prevalence ($p < 0.02$) and mean abundance ($p \leq 0.05$), and not significant ([ns]) for mean intensity. The comparison between *A. simplex s.s.* and hybrids was statistically not significant.

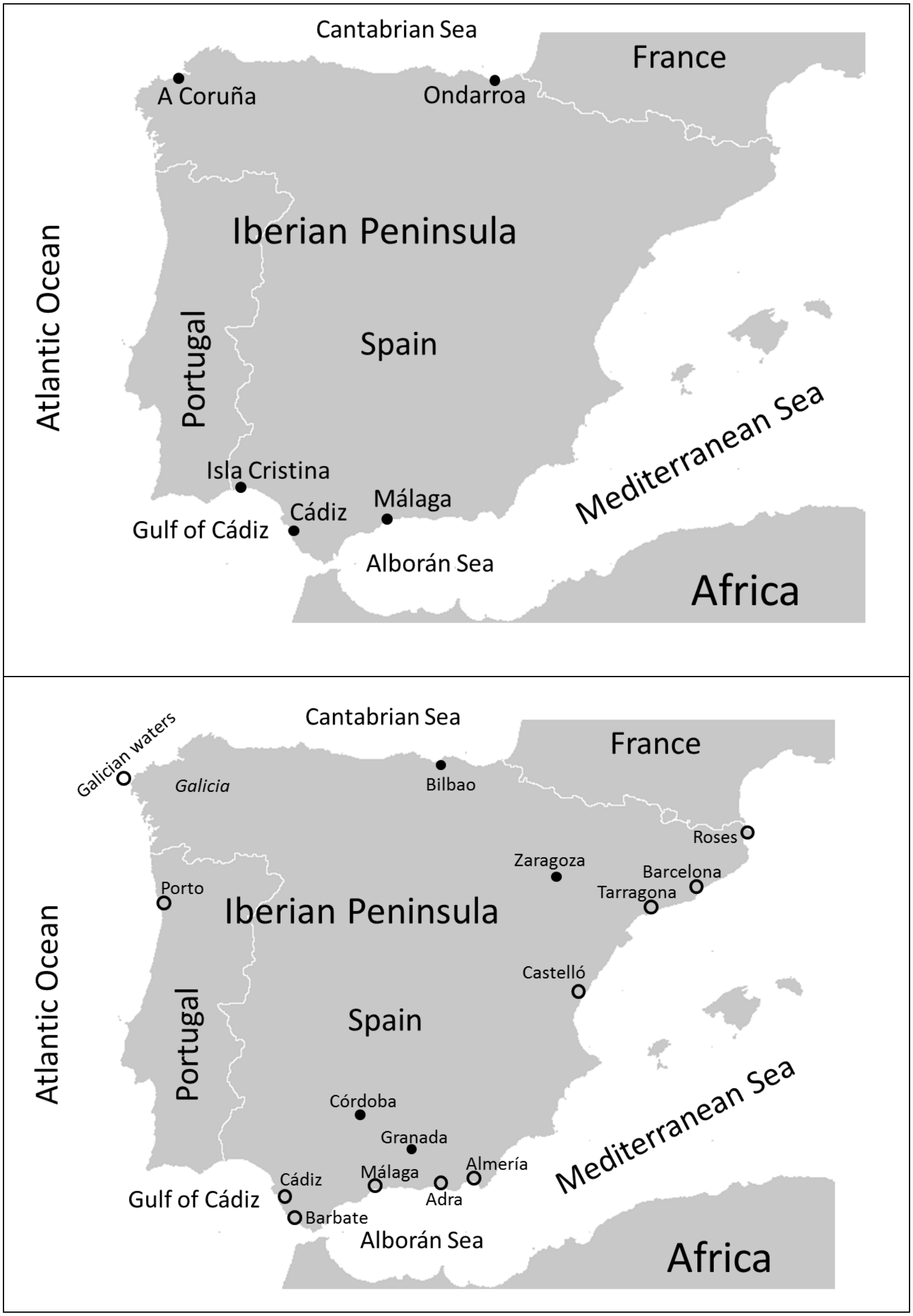

Fig. 1.- Maps of Iberian Peninsula. (Top) Area of investigation showing sampling ports from south and north coasts. (Bottom) Ports (○) and fishmarkets (●) in which the presence of *Anisakis* in sardines has been previously surveyed.

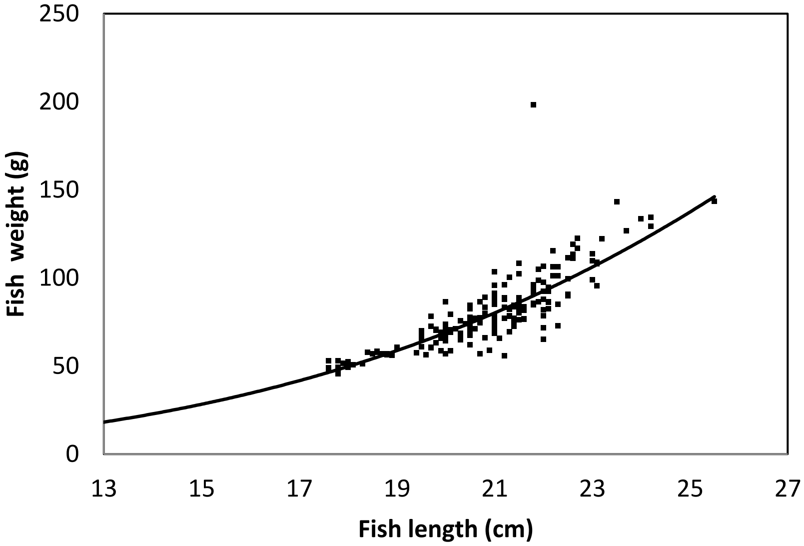

Fig. 2.- Relationship between length and weight in sardines surveyed. The potential relationship is $0.0065x^{3.094}$ with $R^2 = 0.7577$.

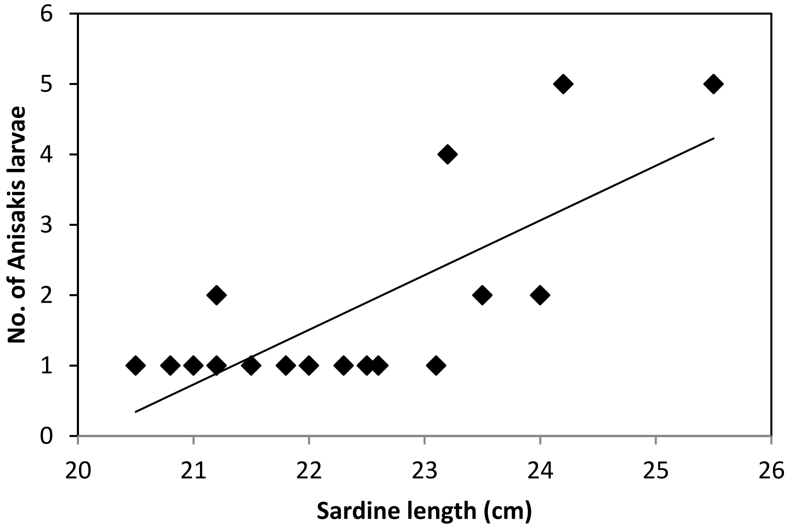

Fig. 3- Intensity of *Anisakis* infection in sardines from A Coruña according their length. The linear relationship is y = 0.7767x – 15.58 with R^2 = 0.5602.