The NE Atlantic European hake: a neglected high exposure risk for zoonotic parasites in European fish markets

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Keywords: hake, epidemiology, zoonotic parasites, risk exposure

ABSTRACT

A sampling programme to understand the factors affecting zoonotic parasite presence and infection intensity of Atlantic European hake populations was conducted from 2013 to 2015. Commercial fish samples comprising 430 hake specimens from northern (Grand Sole) and southern (Atlantic Iberian Peninsula) fishing grounds were sampled, recorded and inspected. We also analysed 75 additional samples collected around Scotland. Parasites were microscopically identified to genus level. Fish biometric measurements were statistically evaluated as categorical predictors of parasite recruitment into fish stocks. Stable isotope composition was determined in another sample batch of 501 hakes from the NE Atlantic (Grand Sole and Galician grounds) to explore trophic indicators as potential predictors of parasite burden. Partial sequences from mitochondrial and nuclear marker genes (mtDNA *cox2* and EF1 α -1 nDNA) from a set of 915 *Anisakis* larvae confirmed the northern stock to be parasitized by *A. simplex* (s.s.) while off the Iberian Peninsula hake there was a mixed infection pattern with larvae of *A. simplex* s.s. (67.4% of identified larvae), *A. pegreffii* (31.9%) and the F1 hybrid (0.7%). The observed burden of *Anisakis* spp. larvae differed markedly between fishing areas. The presence of very high *Anisakis* spp. burdens in a comparatively small proportion of individual fish was well-illustrated with characteristic tensegrity grid architecture for parasite aggregation derived from a plaque-forming cell response observed in the abdominal cavity of larger fish specimens. Demographic infection values were remarkably high in Grand Sole and off the Iberian Peninsula, with 100% prevalence and mean abundances in the fillets ranging from 247 (*A. simplex*) in hake from Grand Sole to 60 *A. simplex* and 20 *A. pegreffii* in southern European populations. Infections were less prevalent and less intense in the hake sample from Scottish waters. Fish size was the most important factor affecting *Anisakis* spp. prevalence and abundance, with larger fish containing more *Anisakis* larvae. *Anisakis* abundance within individual fish was also positively correlated with the condition index although this does not rule out negative effects on the host, and with

trophic level, even after fish size was accounted for. These results suggest that higher *Anisakis* burden is associated with a higher food intake and with eating larger prey. The risk profile for zoonotic parasites herein described for European hake populations underlines the urgent need for adopting a contingency plan that minimizes the risk exposure.

1. Introduction

Hake (*Merluccius merluccius*) is one of the most important demersal fish stocks in European waters, and is commonly caught in mixed fisheries throughout the North East Atlantic and the Mediterranean. There are two stocks of hake in EU Atlantic waters. The northern hake stock is found in the North Sea, Skagerrak, and off the Atlantic coasts of the UK, Ireland and France while the southern stock occurs off the Atlantic coasts of Spain and Portugal. European hake fisheries are of particular economic interest in the North-east Atlantic, supplying fresh high-quality seafood products much prized by regional European markets. In southern European countries with high per capita fish consumption like Spain, hake may represent up to the 30% of the whole seafood protein market provision (MAGRAMA, 2016).

Fishing method, origin and size have been noted as important quality attributes influencing price of European hake (Asche and Guillen, 2012). A number of parasitic diseases described in EU-fish production systems may result in significant economic losses due to reduced marketability of aesthetically unattractive fish, or to rejection by authorities responsible for guaranteeing the health of food animals according to the current legislation. Increasingly, high abundance of visible zoonotic nematodes in fish fillets is seriously affecting the quality of hake sold in European markets (Llarena et al., 2015). Furthermore, increased social awareness of the clinical symptoms of anisakidosis (a human gastric infection which occurs due to ingestion of live parasitic larvae present in raw/undercooked infected fish) and the potential associated allergy (Audicana and Kennedy, 2008), is also being recognized as an important driver of market and consumer choices (e.g. Bao et al., in press; Fernández-Polanco et al., 2013; Mueller Loose et al., 2013).

Several previous studies have investigated the level of *Anisakis* infection in European hake. Valero et al. (2006) and Cipriani et al. (2014) both reported higher levels of *Anisakis* infection in hake from the Atlantic than in the Mediterranean, noting that infection levels were also higher in bigger fish. Ceballos-Mendiola et al. (2010) found that prevalence of *Anisakis simplex* s.s. in hake caught at Little Sole Bank (northeast Atlantic) was 100%. Studies by Sharif and Negm-Eldin (2013) and Ferrer-Maza et al. (2014) both reported a positive relationship between hake size and the number of anisakid larvae present and the latter authors also suggested a negative relationship between overall metazoan parasite burden and reproduction.

Hake is a large predatory fish, with a high mean trophic level (e.g. Korta et al., 2015) and this might be expected to result in a high exposure to *Anisakis*. Although a positive relationship between body size and *Anisakis* burden has been documented in several fish species, there is a lack of studies addressing the relationship between trophic level and *Anisakis* burden. This latter relationship may be complex, reflecting the nature of the predator size – prey size relationship (e.g. Chouvelon et al., 2014) but also the potential for a negative effect of *Anisakis* burden on host trophic level (Britton et al., 2011).

Surprisingly, given the high commercial importance of European hake and published evidence of a high prevalence of *Anisakis* infection, the current strategy for monitoring parasite risk exposure in this species is far from the optimum required to control the risk (Pascual and González, in press). The aim of this study was to define a risk profile for anisakid nematodes of biological and economical importance in European hake populations, quantifying the presence, intensity and spatial distribution of the different anisakid nematode species in hake from different fishing areas of the Eastern Atlantic. The present work reports on part of an epidemiological survey of nematode prevalence and abundance in commercial marine fish in European waters, undertaken under the auspices of the EU FP7 PARASITE project (GA312068). Additional aims were to describe the topological distribution of these parasites in the flesh of European hake, and to explore host-parasite interactions, in particular the relationship between trophic position and *Anisakis* infection. The base-line parasitological data obtained are finally discussed in the framework of international regulations on food safety, focusing on the need for more effective management practices.

2. Material and Methods

2.1. Sampling and parasite detection

A total of 430 European hake (*Merluccius merluccius*) with individual weights from 93 to 3318 g was sampled stratified by season during 2013-2015 in the Grand Sole fishing ground (ICES area VIIj) and in the Atlantic Iberian Peninsula waters (Galician and Portuguese catches from ICES areas VIIIc and IXa, respectively). For the purposes of geographical comparison of infection occurrence and intensity, additional samples of hake were collected opportunistically during 2013 and 2014, from research surveys in Scottish waters (15 fish from ICES area IVa and 60 fish from area VIa). In addition, 501 fish available from three sets of samples (Grand Sole collected by trawling, and two from the Galician coast caught by long-liners and gillnets) were sampled during 2013 for comparison of parasite values in relation to their isotopic signatures (Table 1).

In Spain, fish were sampled during commercial fishing, kept on ice for 24 h on board, and then stored frozen (-20°C) at the lab. Each fish was measured and weighed (Table 1) and

their data were included in the Biobank database (González et al., 2017). Fish were gutted with the aim to separate the edible part. Then, anisakids from the gut were obtained by pepsin digestion (PD) according to Llarena et al. (2013). Both right and left side fillets were divided into four parts: anterior ventral (or belly flaps), anterior dorsal, posterior ventral and posterior dorsal. The UV-press method (Karl and Levsen 2011), with an excitation UV-lamp under 365 nm (power 20 mW), was used to count (and further recover) *Anisakis* larvae from fish fillets. The precise localization of the parasites within the different organs was recorded. In Scotland, samples were obtained from research trawl surveys carried out by Marine Scotland Science and were frozen on-board. Enumeration of *Anisakis* was based on similar methods to those used in Spain except that the UV-press method was used for both fillets and viscera.

In order to permit estimation of the weight if *Anisakis* larvae present in an individual hake, a random sample of 100 worms was weighed.

The presence of leukocytes or macrophages and the degeneration of host tissues in highly parasitized fish specimens were verified, specially, those areas where masses of nematodes were in close proximity to each other. These tissues were fixed with Davidson, embedded in paraffin and processed following standard histological procedures. Tissue sections of 5µm were stained with Masson's trichrome and Hematoxylin/Eosin.

2.2. Parasite identification

After recovery each parasite was identified morphologically under a dissecting microscope to the genus level (Berland 1961, 1991; Smith and Wootten 1984b, 1984a, 1984c). Then, parasite samples and their associated data were entered into an ISO (9001) certified BioBanking Platform which guarantees the traceability and quality of both samples and data (González et al., 2017). Molecular identification of 915 *Anisakis* larvae (408 from viscera and 507 from the flesh) from European Atlantic fishing grounds (ICES areas VII, VIII and IX) was undertaken. The DNA of each individual parasite was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. DNA quality and quantity was checked with a spectrophotometer Nanodrop® ND-2000 (Thermo Scientific). The mitochondrial cytochrome c oxidase subunit II (*cox2*) gene was amplified using the primers 211 F (5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3') and 210 R (5'-CAC CAA CTC TTA AAA TTA TC-3') (Nadler and Hudspeth 2000). PCR reactions were performed in a total volume of 25 µl containing 1 µl of genomic DNA (100 ng), PCR buffer at 1x concentration, 0.3 µM primers, 0.2 mM nucleotides and 0.025 U. µl⁻¹ KAPA Taq DNA polymerase (KAPABIOSYSTEMS). PCR assays were carried out in a Tgradient thermocycler (Biometra), under the following reaction conditions: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min 15 s at 72 °C, followed by 7 min at 72 °C. A negative control (no DNA) was

included in all PCR amplifications. The PCR products were separated on a 2% agarose gel in Tris acetate EDTA buffer, stained with Red Safe and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories). PCR products were cleaned for sequencing using ExoProStar™ 1 Step (GE Healthcare, NJ, USA) following manufacturer recommended protocol. Sequencing was performed in a specialized service and the chromatograms were analyzed using ChromasPro v.1.41 Technelysium Pty Ltd. All generated sequences were searched for identity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA). Additionally, the elongation factor α -1 subunit of nDNA (EF1 α -1 nDNA) was used in all larvae as novel nuclear marker diagnostic of current hybridization between the two cryptic species *A. simplex* (s.s.) and *A. pegreffii* (Mattiucci et al., 2016). The elongation factor (EF1 α -1 nDNA) nuclear gene was amplified using the primers EF-F (5'-TCCTCAAGCGTTGTTATCTGTT-3') and EF-R (5'-AGTTTTGCCACTAGCGGTTCC-3') according to protocol described by Mattiucci et al. (2016). The PCR conditions and procedures for all the anisakid specimens followed those reported in Mattiucci et al. (2016). The sequences obtained at the EF1 α -1 gene for all larvae of *A. pegreffii* and *A. simplex* (s. s.) were compared at the diagnostic positions (i.e. 186, and 286) as detailed in Mattiucci et al. (2016).

2.3. Stable isotope analysis

A representative subsample of flesh was taken from additional samples of hake (ranging from 139 g and 2 kg) caught on Galician and Grand Sole fishing grounds (Table 1) to investigate the relationship between trophic level of hake and parasite burden. Samples were powdered and treated with a 2:1 chloroform:methanol solution to remove lipids and cleaned of surface contaminants. Relative abundances of ^{13}C and ^{15}N were determined by continuous-flow isotope-ratio mass spectrometry. Results are presented in the usual δ notation relative to PDB belemnite and atmospheric N_2 (Air) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. Replicate measurements of internal laboratory standards (albumin, keratin) indicate measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.3\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.4. Statistical modelling

Quantitative descriptors of parasite populations were used as described in Bush et al. (1997). Condition was calculated by using the relative condition factor developed by Le Cren (1951), which is described by the formula:

$$K_{rel} = \frac{W}{aL^b}$$

where W = weight, L = length, and a and b are parameters obtained from the weight-length relationship $\log W = \log a + b \log L$. Since we have no information on length-weight relationships for uninfected hake in the study area, we used the lengths and weights from our sample. For the main analysis we calculated the condition index based on pooled data from all areas. However, preliminary analysis of the length-weight relationships indicated some significant between-area variation - fish from areas VIII (Spanish Atlantic coast) and IX (Portuguese Atlantic coast) tend to be heavier at a given length. We therefore also calculated the condition index using length and weight data from each area separately and investigated whether the models changed if this second index was substituted for the first one. In the pooled sample, the two indices were, as might be expected, highly correlated with each other ($R=0.85$) so only one of them could be used in any given model.

Condition was included alongside length, sex, area (ICES fishery division), year and season as an explanatory variable for the statistical modelling of *Anisakis* presence and abundance in (a) muscle and viscera and (b) in muscle only, and the penetration index (proportion of worms in muscle). We square root-transformed the penetration index to achieve approximate normality. Prior to model fitting we investigated collinearity of the explanatory variables and confirmed that there were no high correlations between them.

In a separate analysis for hake samples with stable isotope data, we examined relationships of *Anisakis* abundance and penetration with fish length, condition, sampling area/gear (three groups, see Table 1), N and C content, and N and C isotope ratios. N and C content were very highly correlated with each other ($R=0.977$) and we therefore dropped C content from the analysis prior to model selection.

Generalized additive models (GAMs) were fitted using backwards selection, based on AIC values and significance of individual variable effects. We assumed a negative binomial distribution for count data (allowing theta to take a value between 0.1 and 10), and used a binomial model for presence-absence. Smoothers describing the effects of continuous explanatory variables were constrained to biologically realistic forms by setting the bases dimension ($k=4$). Model validation included checking that theta values were not on the boundary of the initial range, that there were no influential data points (hat value ≥ 1) and that there were no serious patterns or heteroscedasticity issues in plots of residuals versus explanatory variables. Data exploration, model fitting and model validation were carried out using BRODGAR (Highland Statistic Ltd) and follow Zuur et al. (2007).

3. Results

3.1. Parasite detection and identification

Naked eye visual inspection of the abdominal cavity allowed the macroscopic identification of anisakid larvae. Massive infections (i.e. in fish that were obviously contaminated according to Regulation EC No 853/2004) observed in 38.2% of the hake sampled from Grand Sole and the Iberian Peninsula characteristically presented distinctive masses enclosing numerous larvae of *Anisakis* (Fig. 1). These masses consisted of a connective tissue stroma in which the worms were included. In most of the masses the nematodes were in close proximity to each other, linked by degenerated host tissue and infiltrated with leucocytes, including macrophages. Granulation tissue layers could be seen surrounding many of the nematodes, which further demonstrates the chronic nature of these lesions (Abollo et al., 1998a; Noguera et al., 2009). Otherwise, the UV-press detection method provided dispersed fluoresce images that made it possible to count *Anisakis* larvae from fish fillets.

All sequences obtained from *Anisakis* larvae infecting hakes from Grand Sole (n=253) shared 100% nucleotide identity with sequences of *A. simplex* (s.s) deposited in the GenBank. However, in fish from the Atlantic Spanish-Portuguese coast, mixed infection was found. Specifically, 446 larvae (67.4% of the total) were identified as *A. simplex*, 211 (31.9%) as *A. pegreffii* and 5 (0.7%) were identified as F1 hybrid between both species.

Iberian Peninsula and Grand Sole

Anisakis larvae were highly prevalent in the different batches of fish from the Iberian Peninsula and Grand Sole, reaching an overall prevalence of 99% across these areas (Table 1). The abundance and intensity of infection by *Anisakis* larvae both in viscera and in fillets varied widely among individual fish, ranging from a single parasite to 2148 and 1484 in the viscera and fillets, respectively, and up to 3440 in total. The mean number of *Anisakis* was high in both viscera (152.5) and fillets (128.6), with values up to 90% higher in the Grand Sole ground than in the Ibero-Atlantic Peninsula area. However, it should be noted that the average size of hake sampled at Grand Sole was considerably larger than for the Iberian Peninsula. In hake samples from Scottish waters, prevalence and abundance of *Anisakis* were markedly lower, with very low numbers of larvae recorded in fillets.

Prevalence and intensities were relatively similar between the left and the right fillets in sample from Grand sole and the Iberian Peninsula (Table 1). Between 97 and 99 % of all *Anisakis* were detected in the ventral part of fish fillets, especially in the belly flaps. Similarly, the prevalence of *Anisakis* in the anterior part of the fish was 97% compared to 3% in the posterior part. Only 4 *Anisakis* larvae in total were found in fillets of the 75 fish sampled in Scottish waters, so no conclusions can be drawn about the distribution of *Anisakis* larvae within fillets.

In the Iberian Peninsula samples, the proportion of the different genotypes in the viscera/abdominal cavity (*A. simplex* 68.2%, *A. pegreffii* 30.3%, hybrid 1.5%) was quite similar

to the overall species composition of the infection, although in the belly flaps the proportion of *A. simplex* was considerably higher (89%; *A. pegreffii* 8.2%, hybrid 2.8%).

Within the Iberian samples, there was a marked difference in the prevalence of *A. pegreffii* with very low prevalence in ICES VIII (6.7% in viscera and 14.2% in fillets) and highest abundance in ICES IX (from 85 to 93% in viscera and fillets, respectively). The species *A. pegreffii* was absent in hakes from area ICES VII. Regarding *A. simplex*, it was present in hake from all NE Atlantic areas regardless of the site of infection.

While *Anisakis* larvae may be present in large numbers they contribute little to the overall weight of the fish. A sample of 100 larvae weighed 0.168 g, i.e. an average of 0.00168 g. This suggests that even the most severe infection (3440 worms) thus represents a total weight of only around 5.7 g in a fish of 942 g, or approx. 0.6% of total body weight.

3.2. Factors affecting parasite recruitment

Main analysis for all areas

For the analysis of *Anisakis* presence, the explanatory variable area had to be dropped since two areas had uniform values for presence (all 0 in the North Sea and all 1 at Grand Sole). Furthermore, in the initial model there was no significant sex effect and this variable was dropped due to the higher number of missing values, which reduced sample size from 505 to 427. In the model for *Anisakis* presence in viscera plus fillets, both year and condition were dropped during model selection due to non-significant effects. The final model included a positive linear effect of fish length and showed higher prevalence in spring and summer than in winter. Evidently (see Table 1) there is also a strong area effect but, as noted above, it could not be included in the statistical model. In the case of *Anisakis* in fillets, the final model included a positive effect of length (linear in the mid-range of lengths and with wide confidence limits in the smallest and largest fish) and indicated higher prevalence in spring and summer than in winter and in 2013 than in 2014. It should be noted however that, given the opportunistic sampling regime, area, year and season effects could be confounded, especially if one of these variables is necessarily excluded from the analysis.

Raw data on abundance of *Anisakis* in viscera plus fillets and in fillets are displayed in Fig. 2., which clearly shows that abundance increases with length and was generally lower in the Scottish samples than in the other samples. Furthermore, there is a suggestion that, for their length, Spanish samples were characterized by higher abundance than seen in Portuguese or Grand Sole samples. For the statistical analysis of abundance, because *Anisakis* was absent in fillets of fish sampled in the North Sea, samples from this area were deleted from the analysis of abundance in fillets. The initial models for abundance of *Anisakis*, in (a) viscera and fillets and (b) in fillets only, both included significant effects of sex ($P=0.0061$

and $P=0.0091$ respectively), with higher abundance in males than in females. However, because of missing values, this also reduced overall sample size substantially and we therefore continued model fitting without including sex. In the first model, for abundance in viscera and fillets, the year effect was non-significant and we dropped this from the final model. All remaining effects were significant. *Anisakis* numbers increased with fish length up to lengths of around 500 mm (Fig. 3a). Condition had a linear positive effect (Fig. 3b), numbers were higher in quarter 1 than in quarters 2 or 3, lower on the west coast of Scotland than in the North Sea, and higher in Grand Sole and the Iberian Peninsula than in the North Sea. In the second model, for abundance in fillets only, all explanatory variables were retained. Effects of fish length and condition were very similar to those in the first model: abundance was higher in quarter 1 than in quarter 3, and higher in Grand Sole and the Iberian Peninsula than on the west coast of Scotland. Abundance was also higher in 2014 than in 2013 (see Table 2 for details).

In the analysis of (square root transformed) penetration of *Anisakis* into fish fillets, the initial model showed no significant effect of sex and this variable was dropped first as its inclusion reduced sample size. Following further model selection, the final model included effects of area, length and condition. Penetration initially increased with increasing fish size but tended to be lower in the largest fish (Fig. 3c). The effect of condition was weakly positive (Fig. 3d). Penetration was higher in Grand Sole and the Iberian Peninsula than in Scottish waters.

When models for *Anisakis* abundance were re-run using area-specific condition indices, i.e. based on applying the Le Cren index only within each fishery area, length and condition effects in the resulting models did not change substantially. The condition index had been dropped from the final models *Anisakis* presence due to having no significant effect. However, using the area specific condition index, condition had a weak linear positive effect on *Anisakis* presence in flesh ($P=0.0193$). The equivalent effect in the model for *Anisakis* presence in viscera plus flesh was not significant ($P=0.0718$). Finally, re-running the model for penetration of *Anisakis* into flesh, using area-specific condition slightly increased goodness of fit (%deviance explained rose from 55.5% to 56.5% and the smoother for the partial effect of condition reached an asymptote at index values of around 1.0).

All model fits were satisfactory in terms of an absence of influential data points, and absence of patterns in residuals.

Analysis for fish with isotope data

Since prevalence was 100% in these samples, no models of *Anisakis* presence were possible. The final model for *Anisakis* abundance in the viscera and fillets of the hake sampled for stable isotope analysis included effects of fish length (positive), N ratio (or $\delta^{15}\text{N}$) (weakly positive), C ratio (or $\delta^{13}\text{C}$) (a peak at intermediate values and clearly decreasing at high values) and

sample identity (higher in Grand Sole trawls than in Galician coastal gillnets) (Table 3, Fig. 4). A very similar model resulted for *Anisakis* abundance in fillets (Table 3), although the effect of N ratio in this model was marginally non-significant ($P=0.067$). The final model for the penetration index also included effects of area, length, N ratio and C ratio. The length effect was negative and the N and C ratio effects were approximately U and $\cap$ -shaped respectively (Table 3, Fig. 5).

4. Discussion

4.1. Parasite identification

Based on the use of mitochondrial and nuclear gene markers the genotypes identified from the hake sampled in the northeast Atlantic corresponded with the species *A. simplex* and *A. pegreffii*, and the F1 hybrid between both species. This result is consistent with previous studies, which indicate Atlantic waters of the Iberian Peninsula waters as a hybrid zone for the two sibling species (Abollo et al. 2003). Mixed infections of *A. simplex* and *A. pegreffii* have also previously been reported in hake from several northeast Atlantic locations, with the former species dominating except off Morocco (Mattiucci et al., 2004; Cipriani et al., 2014). Our results also reinforce the idea of a latitudinal gradient in the occurrence of both parasite species. In the Grand Sole only *A. simplex* s.s. were found. The prevalence of *A. simplex* decreased towards the south (it was lowest in Portuguese waters) alongside a corresponding increase in the prevalence of *A. pergreffii*. Mattiucci et al. (2004) found no *A. simplex* in Mediterranean hake samples - *A. pergreffii* was generally the most abundant *Anisakis* species although a third species, *A. physeteris*, was more abundant in some western Mediterranean sites. Evidently, *Anisakis simplex* and *A. pergreffii* may coexist in the same individual fish and are sympatric species across a wide range of latitudes within Northeast Atlantic fishing grounds.

4.2. Epidemiology

It is worthy of note that demographic infection values for *Anisakis* spp. in hake from three of the studied areas are by far the highest ever recorded in any EU-fish production system: 100% prevalence and abundance equivalent to 25-50 parasites/100 g of total weight in Grand Sole (ICES area VII), 10-25 in Spanish Atlantic coastal waters (VIIIc) and 5-10 in Portuguese coastal waters (IXa). These high values may be partly explained by the way the data were collected, namely the UV-press method, an effective (albeit invasive) method for parasite detection, which has been validated under a ring trial involving highly experienced laboratories (Gomez-Morales et al., in press), and permits accurate and precise counting of the total

number of anisakids present in the edible part of fish. The parasite counts from fillets were added to parasite counts from the viscera to give a reasonably complete picture of the total count of *Anisakis* spp. in each infected hake.

Setting aside methodological improvements, the relatively high trophic level (mesopredator status) of hake in NE Atlantic ecosystems presumably greatly contributes to the heavy infections observed in some areas. Small hake mostly feed on species from the mesozooplanktonic community (mainly euphausiids of *Nyctiphanes couchii*; Gregori et al., 2015), subsequently shifting to a piscivorous diet. Then, smaller hake prey on small pelagic fish, while larger hake take larger demersal prey such as blue whiting (Mahe et al., 2007). All these prey species are infected by anisakid nematodes which are thus transmitted to the hake. Hake is in turn an important prey for dolphins (Santos et al., 2014), top predators which play a crucial role in maintaining the parasite life cycle in the sampled areas (Abollo et al., 1998b).

The analysis of *Anisakis* presence and abundance in hake was based on generalized additive modelling, in principle capable of separating the partial effects of multiple explanatory variables, both continuous and categorical, and allowing non-linear relationships. However, the power to separate these effects – and to look for interactions – depends on the sampling. While the opportunistic sampling approach used in the present study provided good coverage of a range of body sizes from several areas, coverage of the study period (mainly 2013-2014) was patchy and caution is therefore needed, especially in interpreting apparent effects of season and year.

Samples collected in Scottish waters had relatively low prevalence and intensity of *Anisakis* infection, although it should be noted that sample sizes were smaller, especially in the North Sea, and they included relatively few large hake – although it was notable that *Anisakis* was absent even in some fish of up to 450 mm TL. We cannot completely rule out a methodological component to this difference, in that the UV-Press method was also applied to viscera in the Scottish study. However, visual examination was thorough and a member of the Scottish team (LCH) took part in a PARASITE training event for use of the UV-press method. It is therefore not thought that many worms would have been missed and, in any case, the UV-press method was used for the fillets in all study areas.

As documented in many fish species (see Adroher et al., 1996; Bao et al., 2015; Bratley and Bishop, 1992; Chou et al., 2011; Levsen and Lunestad, 2010; Levsen et al., 2016; Mladineo et al. 2012; Munster et al., 2015; Zuo et al., 2016), hake in the present study became far more infected as they increase in size, presumably reflecting a combination of increased food intake and accumulation of worms - as the *Anisakis* larvae are apparently retained for prolonged (although as yet undefined) periods. A positive relationship of *Anisakis* abundance with hake size was also reported in several previous studies on European hake (Cipriani et al., 2014; Ferrer-Maza et al., 2014; Sharif and Negm-Eldin, 2013; Valero et al., 2006). It should

however be noted that this is not a universal phenomenon in fish: some studies either found no relationship between fish size and *Anisakis* infection or even a negative correlation (Horbowy et al., 2016; Levsen and Berland, 2011; Rello et al., 2008; Stromnes and Andersen, 1998).

The statistically significant positive relationship of *Anisakis* infection abundance with fish condition suggests that the dominant process involved may relate to feeding intensity: a higher food intake results in both better condition and a higher *Anisakis* burden. However, this may be over-simplistic. Some of the sampled hake showed clear evidence of a defensive reaction to the presence of *Anisakis*. The immune response resulted in the formation of free parasite tissue masses in the visceral cavity. It is most likely that these masses had broken away from the surface of the viscera and maintained their integrity via continuing host response and formation of tissue adhesions between parasites as well as the visceral cavity (Fig. 1). This phenomenon is well known in salmonid aquaculture, where the combination of antigens and the adjuvant elicits similar adhesions within the visceral cavity (Bahlool et al., 2012; Noguera et al., 2009). The mass of these adhesions is probably significantly greater than the mass of the worms alone and its inclusion in the calculation of the condition index may thus also give a misleading impression of condition.

The statistical modelling also suggested the existence of higher abundance (but not prevalence) of *Anisakis* in male hake, as well as some seasonal and between-year differences in prevalence and abundance. Given that we do not have samples for all seasons in both years in all areas, we cannot reach any firm conclusions about the nature of temporal variation in infection levels, other than that it exists. Rather than implying fluctuations in individual fish this probably reflects fish movements (i.e. we are not always sampling the same components of the population in a given area).

In the second analysis, with stable isotope data, significant effects of stable N and C isotope ratios (although not element concentration) were evident. The nitrogen isotope ratio is generally viewed as representing trophic level. The weak positive relationship between total *Anisakis* numbers and the nitrogen isotope ratio is consistent with fish at a higher trophic level accumulating more worms. The effect may be weak since it is a partial effect, with the effect of body length (also expected to correlate with trophic level) already being accounted for.

The relationship of parasite numbers with the carbon isotope ratio showed a peak at intermediate $\delta^{13}\text{C}$ values. The carbon isotope ratio can be an indicator of inshore-offshore habitat (Hobson et al., 1994), in which case the sharp decline in *Anisakis* numbers at higher carbon isotope ratio values could suggest lower infection in fish from inshore waters. There is also typically latitudinal variation in carbon isotope ratios, with lower $\delta^{13}\text{C}$ values at higher latitudes in a range of taxa including marine phytoplankton (Rau et al., 1982). However,

latitudinal trends in *Anisakis* infection are probably already captured by including fishing area in the model.

Slight seasonal and/or ontogenetic shifts in trophic level (as indicated by the isotope signal) occupied by hake may obviously contribute to the heterogeneity in the distribution pattern observed in parasite recruitment. Other factors such as trophic connectivity between intermediate, paratenic and final hosts, and oceanographic conditions will also contribute to the infection levels seen at each fishing ground (Pascual et al., 2007).

Regarding the edible part of fish, no significant differences were observed in the number of *Anisakis* between the right and left fillets from all sampled areas. However, clear patterns were seen in relation to infections in the posterior/anterior and dorsal/ventral parts of the fish muscle. In general, the antero-ventral part (belly flaps) concentrated most of the parasite burden of a given infected fish, regardless of the fishing ground. Similar results have previously been obtained for *Anisakis* infections in several fish species, including herring (Levsen and Lunestad, 2010), cod, monkfish, mackerel, herring (Petrie et al., 2007) and seabass (Bernardi et al., 2011). It seems likely that the proximity of the fish gut and visceral cavity to the antero-ventral musculature facilitates the migration of worms toward this part of the fish but other factors (e.g. related to the fish immunological response, condition, density-dependent mechanisms and aggregating behavior) may also contribute to this pattern.

The tissue specificity of the two sibling species indicates that the more prevalent *A. simplex* has the highest penetration index in the body muscle, although both *A. pegreffii* and the F1 hybrid have also shown a high penetration capability into the belly flaps. Knowledge of the abundance of the different genotypes herein reported is of clinical relevance regarding the differential pathogenic potential of different species (Arizono et al., 2012).

4.3. Risk management

European hake (*Merluccius merluccius*) is among the most important commercial species in fish markets along the Atlantic coast of southern Europe. Although several studies have dealt with the presence of zoonotic parasites in this species, very few data were previously available on the factors affecting the infection parameters and topology. Public health authorities have a stake in, and responsibility for, the control of fish zoonoses originating from fisheries. The ability to control biohazards of a parasitic nature in fishery products relies on the shared capacity of authorities and fishing/food sector to detect emergent hazards/episodes early and to rapidly implement control measures. In fact, a broad Regulation Operational Framework at the EU and Member Countries level (including a set of EFSA and National Food safety authorities Opinions and recommendations) was established in 2004 to tackle the challenges posed by parasite hazards in fishery products. Despite this effort, overall epidemiological

values of about 14 infective larval forms per serving (i.e., per 100 g fish fillet), herein reported for commercial fresh hake that potentially reach markets, which is responsible for emergent anisakiasis (through consumption of undercooked hake products) and associated allergies. Related to this issue, there was an interesting debate in terms of the allergenic potential of dead larvae, which in the past was not given for granted (Alonso-Gómez et al., 2004; Daschner et al., 2012). However, the presence of thermostable parasite allergens that could potentially provoked reactions to previously sensitized patients was actually proved (e.g. Carrera et al., 2016). The parasite appears as an important agent for chronic urticarial and in endemic countries, the amount of highly sensitized subjects in the general population could be as high as 7%. Adequate information to asymptomatic patients on fish consumption habits would avoid new contacts with parasite allergens and decrease their specific IgE levels and consequently the appearance of acute or chronic episodes induced by the parasite (Moneo et al., 2017).

The above statements do not mean that veterinary services and fishing organizations are not complying with seafood safety laws and recommendations but the performance of the latter is called into question by rapid advances in risk assessment and new integrated tools. Essentially, control practices along the seafood supply-chain do not currently guarantee that hake products are parasite-free when they reach the end-users. In fact, the seafood industry and consumers have to deal with an increasingly level of exposure to parasite risk, usually without access to appropriate monitoring equipment, education or training to ensure the inactivation of infective zoonotic larvae and their allergens (Audicana et al., 2002; Audicana et al., 2008; Rodríguez-Maillo et al., 2010; Baird et al., 2014; Carrera et al., 2016; Moneo et al., 2017). The transposition of tasks/competences introduced in the new provisions by the EU Food Hygiene Package (Regulations 852/2004, 853/2004 and 854/2004), whereby official veterinarian inspections are replaced by Hazard Analysis Critical Control Point (HACCP) controls made by fish operators, has introduced a high level of uncertainty for the fishery sector, which now has to deal with customer and consumer complaints under a rather ineffective regulatory framework which states that any parasitized fish stuff should be considered unfit for human consumption, but fails to achieve this.

Solutions for ensuring healthier and more appetizing hake in EU production systems require a deeper understanding of complex interactions between the marine ecosystem and people, ideally a “food system analysis” approach, considering how production, distribution and consumption practices contribute to exposure to the risk profile, i.e. exposure of consumers of hake products to parasite hazards. There is a need for (1) scaling-up the parasite control in the seafood supply chain (adapted to regional and country conditions) with consistent and reliable surveillance and reporting (from the net to the plate) and (2) linking epidemiological information to food safety programs and risk communication policies, to both

strengthen the competitiveness of seafood industry and guarantee the public health protection of EU consumers.

The establishment of a common framework for governance and action to detect, monitor and control of parasitic zoonotic diseases in seafood requires the coordination of many actors in the seafood chain (administrations, producers, businesses in the fish industry, civil society and R&D partners). In order to achieve effective management of the problem, it is important to design upgraded hazard analysis and critical control points for each specific parasite hazard, on a case-by-case basis, considering the nature of the problem (especially the type of pathogen and the diagnostic criteria), the epidemiological surveillance programs, disease control and the effectiveness of response options/available treatments for various fish products. Cost-benefit analysis is needed to evaluate the feasibility of specific management options.

In the case of Atlantic European hake two aspects are of particular concern: firstly, intra-vitam migration of *Anisakis* larvae into the flesh is recognizable by the high number of melanized parasitic capsules in the flesh; secondly, topological evaluation of infection reveals an aggregated distribution of *Anisakis* larvae with a high concentration in and around the abdominal cavity, especially the belly flaps (see Fig. 1). Firstly, therefore, evisceration should be improved, including the possibility of removing the belly flaps from the final product.

Acknowledgements

This research was supported by the European Commission under the project PARASITE (FP7-GA-312068). We would like to thank S. Feist and A. Sitja-Bobadilla for their advice on *Anisakis* grids. Michaela Paoletti, Valentina Nardi (UT-URS), J.M. Antonio, H. Rodríguez and J. Tamame (IIM-CSIC) provided valuable technical assistance. Miguel Bao, Anna Dunser and Ken Mackenzie assisted with data collected in Scotland.

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Table 1. Sample data for European hake used in (a) epidemiological and (b) isotopic analyses.

(a) Epidemiological survey				Fillets					Viscera	
Origin	N	TL	TW	P(%)	A and I	V:D	L:R	A:Po	P (%)	A and I
West coast of Scotland (winter 2014) (VI)	60	403±60 (321-644)	452±257 (195-1690)	5.0	A: 0.1 (0-2) I: 1.3 (1-2)	100:0	25:75	75:25	56.7	A: 3.6 (0-58) I: 6.3 (1-58)
North Sea (autumn 2013, winter 2014) (IV)	15	325±156 (80-601)	365±400 (2-1490)	0.0	A: 0 (0-0) I: -	-	-	-	46.7	A: 12.3 (0-72) I: 26.3 (1-72)
Grand Sole (spring 2013, winter 2014) (VII)	188	533±83 (330-785)	940±532 (230-3318)	100	A: 247.1 (6-1484) I: 247.1 (6-1484)	99:1	53:47	97:3	100	A: 289.6 (19-2148) I: 289.6 (1-2148)
Galicia (autumn 2013 + spring 2014) (VIII)	99	341±56 (255-570)	268±195 (93-1472)	95.9	A: 60.1 (0-665) I: 62.6 (2-665)	98:2	54:46	98:2	96.0	A: 66.3 (0-782) I: 69.1 (2-782)
Portugal (spring 2013 + summer 2014) (IX)	143	353±55 (275-600)	303±202 (141-1635)	83.9	A: 20.1 (0-486) I: 24.0 (1-486)	97:3	52:48	97:3	97.2	A: 32.5 (0-862) I: 33.4 (1-862)
(b) Stable isotope survey										
Grand Sole (October 2015) (VII) [Trawlers]	85	529±81 (380-780)	1017±537 (322-3540)	100	A: 345.5 (6-1484) I: 345.5 (6-1484)	-	-	-	-	A: 335.8 (19-1673) I: 335.8 (19-1673)
Galicia (October 2015) [longliners]	69	333±30 (275-420)	261±81 (148-564)	100	A: 20.6 (2-292) I:	-	-	-	-	A: 28.9 (2-256) I: 28.9 (2-256)
Galicia (October 2015) [gillnets]	347	610±91 (375-1045)	1786±905 (348-9720)	100	A: 162.3 (2-845) I: 162.3 (2-845)	-	-	-	-	A: 354.2 (3-3996) I: 354.2 (3-3996)

Sampling areas (with ICES fishery division in parentheses) and seasons (quarters of the year) are indicated. Abbreviations: TL – Total body length (mm; mean ± standard deviation and range in parentheses), TW – Total body weight (g; mean ± standard deviation and range in parentheses). P (%) – Prevalence (as a percentage), A – Abundance (mean with range in parentheses), I – Intensity (mean with range in parentheses), following the terminology of Bush et al. (1997). Ratios for the percentage of worms located in different parts of the fillet, summed across all samples: V:D – ventral portion (corresponds roughly to belly flap) *versus* dorsal, L:R – left *versus* right, A:Po – anterior *versus* posterior.

Table 2. Summary of GAM results for presence and number of *Anisakis* in whole hake and in hake muscle, and penetration into fillets (all samples). Statistical significance of effects of explanatory variables is classified as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$. %DE = Percentage of deviance explained by the final models (after backwards selection). Dropped = removed during backwards selection. Notes: sample size for the penetration index is lower as the index is undefined when *Anisakis* is not present. Fish sex was initially included as an explanatory variable (see results in main text) but dropped due to the large number of missing values. The explanatory variable area could not be included in analysis of presence and North Sea (area IV) samples were excluded for analysis of abundance in muscle, in both case due to all fish in at least one sample having identical values of the response variable..

Response	N	Area	Length	Condition	Season, Year	%DE
Presence <i>Anisakis</i> (binomial)	505	Not included	***, positive	Dropped	Year: dropped Season ***, Q1 < Q2, Q3	49.9
Presence <i>Anisakis</i> in flesh (binomial)	505	Not included	***, mainly positive	Dropped	Year: ***, 2013 > 2014 Season ***, Q1 < Q2, Q3	47.9
Count <i>Anisakis</i> (neg bin)	505	***, VI < IV < VII, VIII, IX	***, positive, with asymptote	***, positive	Year: dropped Season ***, Q1 > Q2, Q3	76.7
Count <i>Anisakis</i> in muscle (neg bin)		***, VI < VII, VIII, IX	***, positive, with asymptote	**, positive	Year: ***, 2014 > 2013 Season: ***, Q1 > Q2, Q3	75.0%
Penetration index (sqrt transformed)	462	***, IV, VI < VII, VIII, IX	***, $\cap$ -shaped	*, positive	Year: dropped: Season: dropped	55.5

Table 3. Summary of GAM results for abundance of *Anisakis* and the penetration index in viscera plus fillets and in fillets of hake sampled for stable isotope ratios. This analysis was carried out on separate samples from Grand Sole and Galicia. Effects of sample source, fish length and condition were also tested. Statistical significance of effects of explanatory variables in the final models (after backwards selection) is classified as: . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$. %DE = Percentage of deviance explained by the model GST = Grand Sole, trawl, GCG = Galicia coast, gillnet. Dropped = removed during backwards selection.

Response	N	Group	Length	Condition	N content	N ratio	C ratio	%DE
Count <i>Anisakis</i> (neg bin)	501	***, GST > GCG	***, positive	Dropped	Dropped	*, Positive	***, ∩-shaped	54.1
Count <i>Anisakis</i> in flesh (neg bin)	501	***, GST > GCG	***, mainly positive	Dropped	Dropped	., Weakly positive	***, ∩-shaped	43.1
Penetration index (sqrt transformed)	501	***, GST > GCG	***, negative	Dropped		*, U - shaped	*, ∩-shaped	24.2

Fig. 1. European hake belly flap linings covered by nematode masses (left) under a visual inspection scheme. Haematoxylin-eosin stained cross-sections of nematode masses, showing several encapsulated *Anisakis* larvae in close proximity forming a tensegrity grid as a result of a vigorous inflammatory response in the fish (right).

Figure 2. Log-transformed abundance of *Anisakis* in (a) viscera and fillets and (b) fillets of hake plotted against fish length and distinguishing samples from different areas.

a

b

c

d

Figure 3. Smoothers showing the partial effects of (a) total length (TL) and (b) condition on numbers of *Anisakis* in hake viscera plus fillets, and partial effects of (c) total length and (d) condition) on penetration of *Anisakis* into fish fillets (all sample groups considered).

Figure 4. Smoothers showing the partial effect of (a) total length, (b) N isotope ratio and (c) C isotoperatio on numbers of *Anisakis* in hake muscle and viscera.

a

b

c

Figure 5. Smoothers showing the partial effect of total length, N ratio and C ratio on penetration ratio of *Anisakis* in hake.