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

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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 F1hybrid (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

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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 simples 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-Reino 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. This tissues were fixed with Davidson, embedded in paraffin and processed following standard histological procedures. Tissue sections of 5 µm 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; Smith and Wootten 1984a, 1984b, 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 ml containing 1 µl of genomic DNA (100 ng), PCR buffer at 1 x 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 ExoProStarTM 1 Step (GE Healthcare, NJ, USA) following manufacturer recommended protocol. Sequencing was performed in a specialized service and the chromatograms were analysed 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