Comparison of anisakid infection levels between two species of Atlantic mackerel (Scomber colias and S. scombrus) off the Atlantic Portuguese coast

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Summary: Anisakiasis is a problematic zoonotic infection associated with the consumption of raw or undercooked fish. Atlantic mackerel (Scomber colias) is of high commercial interest in Portugal and has been reported as a common host of Anisakis spp. In this study, the occurrence of anisakids is evaluated in S. colias and also Scomber scombrus, and the potential zoonotic risk associated with consumption of these two fishes is evaluated according to the recorded infection levels. These were found to be high for both fish species: a mean intensity and prevalence of 21.7 worms/fish and 85% for S. colias, and 16.4 worms/fish and 83.3% for S. scombrus, respectively. No correlation was detected between anisakid intensity and host total length, total weight, condition factor, and hepatosomatic and gonadosomatic indices for both fish species, but significantly higher intensity values were detected for more mature S. scombrus, i.e. fish recording a higher gonadosomatic index. Molecular tools allowed the identification of two species of Anisakis, A. simplex (s.s.) and A. pegreffii. They differed in their occurrence; in S. colias the prevalence of A. simplex (s.s.) was 18% and that of A. pegreffii was 82%, whereas in S. scombrus the prevalence of A. simplex (s.s.) was 73% and that of A. pegreffii was 27%. Occasionally, worms of Hysterothylacium aduncum were identified for both fish. The different infection levels of the two Anisakis species in both hosts off the Portuguese coast raise the hypothesis of a different life cycle at the level of the invertebrate intermediate host. S. colias lives in deeper waters than S. scombrus, and the differences found in infection levels suggest that A. pegreffii main first intermediate host also live in deeper waters, compared with A. simplex (s.s.) main first intermediate host. The higher infection levels of A. simplex (s.s.) (most infectious to humans) in S. scombrus suggest that its consumption when slightly cooked, as in grilled fish (so popular in Portugal), could be more problematic for the development of anisakiasis in humans than the consumption of S. colias and thus be of potential public health concern.

Keywords: anisakids; Anisakis pegreffii; Anisakis simplex (s.s.); Atlantic mackerels; Portuguese coast; molecular identification; food safety.

Resumen: Anisakiasis es una zoonosis problemática asociada con el consumo de pescado crudo o poco cocinado. La caballa del Atlántico (Scomber colias) es un pez de mayor interés comercial en Portugal y se ha informado que es un hospedador común de Anisakis spp. En este estudio, la incidencia de anisáquidos se evaluó en S. colias y Scomber scombrus, y el potencial riesgo zoonótico asociado con el consumo de estos dos peces se evaluó de acuerdo a los niveles de infección registrados. Estos se encontraron altos para ambas especies de peces - intensidad media y la prevalencia de 21.7 gusanos/pescado y 85% para S. colias, y 16.4 gusanos/pescado y 83.3% para S. scombrus, respectivamente. No se detectó ninguna correlación entre la intensidad de anisáquidos y datos del hospedador, longitud total, peso total, factor de condición, y los índices hepatosomáticos y gonadosomáticos para ambas especies de peces, pero sí se detectaron valores de intensidad significativamente más altos para los pescados más maduros en S. scombrus, es decir, de pescado con mayor índice gonadosomático. Las herramientas moleculares permitieron la identificación de dos especies de Anisakis simplex, es decir, A. simplex (s.s.) y A. pegreffii. Ambos diferían en su ocurrencia – la prevalencia de A. simplex (s.s.) fue del 18% y del 82% A. pegreffii en S. colias, mientras que la prevalencia de A. simplex (ss) fue del 73% y del 27% A. pegreffii en S. scombrus. De vez en cuando, se identificaron los gusanos de Hysterothylacium aduncum para los dos peces. Los diferentes niveles de infección de las dos especies de Anisakis en ambos hospedadores, frente a la costa portuguesa, plantean la hipótesis de un ciclo de vida diferente en el nivel del huésped intermediario invertebrado. S. colias vive en aguas profundas frente a S. scombrus, que podría ser también cierto para...
INTRODUCTION

Portugal has a long tradition of fishing and is the leading country in fish consumption in the European Union (EUMOFA 2016), with 55.3 kg of sea food per capita per year in 2014. For this reason, zoonotic infections such as anisakiasis are a potential risk and a case of major public health concern in this country. Fish dishes, such as grilled (slightly cooked) fish, are one of the most popular food specialties in Portugal, not only among the local people but also among the many tourists who visit the country each year. Atlantic mackerels Scomber colias Gmelin, 1789 and Scomber scombrus L., 1758 are pelagic-neritic scombrids of great commercial interest that are consumed lightly grilled. According to the Portuguese Institution for Statistical Data, 29543 t of S. colias and 588 t of S. scombrus were landed in 2014 (DGRM 2015), with S. colias being one of the top four fish sold in the country.

Anisakid nematodes are the most abundant parasites of marine fishes worldwide (Mattucci and Nascetti 2008). They are parasites of zoonotic potential, having the ability to infect humans through the consumption of raw or lightly cooked fish, causing the emerging infection anisakiasis, and thus being a food safety concern (MacCarthy and Moore 2000). Associated symptoms of anisakiasis include severe gastric or intestinal disease or, in the mild version, allergies (Audicana et al. 2002, 2003). In Portugal, no reports of severe disease due to Anisakis infections have been recorded so far. However, in a survey conducted among the population in a fishery town located in the south of the country, it was found that 8% of the people were allergic to anisakids (Nunes et al. 2003). In Spain, France, Italy, Germany, the Netherlands and Japan, severe episodes of anisakiasis have already been recorded (Arizono et al. 2012, Mattucci et al. 2013, Ubeira et al. 2000).

Both S. colias and S. scombrus are common hosts of anisakids in different geographic areas, particularly in Europe (Abollo et al. 2001, Mattucci and Nascetti 2008, Pontes et al. 2005). There are also several records from African countries (Abattouy et al. 2011, Farjallah et al. 2008, Kijewska et al. 2009). So far, no updated information is available for Atlantic mackerels fished off continental Portugal, and no attempt has been made to identify the anisakids isolated from S. scombrus to the species level (Rego et al. 1985).

The main purpose of this study was to determine and compare the infection levels (prevalence and intensity) of anisakid nematodes in S. colias and S. scombrus, to correlate parasite and host data, and to evaluate whether the fish species can represent a danger when consumed raw or undercooked, taking into account (i) the infecting species and (ii) the recorded infection levels.

MATERIALS AND METHODS

Host sampling

Several samples of Atlantic chub mackerel, S. colias (n=40 in total), and Atlantic mackerel, S. scombrus (n=42 in total), were collected from October to December 2009 (n=19 and 21, respectively) and from January to June 2010 (n=21 for both species). The Atlantic mackerels were fished in the FAO area Atlantic Northeast 27, subarea Portuguese waters IX, by trawling fisheries operated by local boats. The fish were all purchased at the harbour fish market and then freshly dissected or frozen for subsequent parasitological analyses. Host identification was done according to Collette (1986), based on the external and internal features. Fish length was also evaluated [mean±standard deviation (range)]: 32.6±3.6 (25.4-39.2) cm for the Atlantic chub mackerel; and 31.9±2.3 (21.8-36.4) cm for the Atlantic mackerel (see Table 1). As some parasites might accumulate in older, i.e. larger fishes, and thus bias our sample, a set of hosts with similar lengths were used for the analysis. Also, fish weight (g), Fulton’s condition factor (K=weight/length^3 g cm^-3), hepatosomatic index (HI=leber weight (g) / total weight (g) × 100), gonadosomatic index (GI=gonadal weight (g) / total weight (g) × 100) and sex ratio were recorded for each fish species (see Table 1).

Parasitological survey

During the parasitological survey for anisakids, the following infection sites were analysed under the
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Table 1. – Comparison of host data (total weight, total length, condition factor, hepatosomatic index, gonadosomatic index and sex ratio) and anisakid infection levels (prevalence and intensity) in the Atlantic mackerels, *Scomber colias* and *S. scombrus*, off the Portuguese continental coast. * mean±SD (minimum-maximum)

|                      | *Scomber colias* (n=40) | *Scomber scombrus* (n=42) |
|----------------------|-------------------------|---------------------------|
| **Host Data**        |                         |                           |
| Total weight (g)*    | 346.3±128.4 (135.3-602.4) | 288.9±73.0 (62.5-474.5)   |
| Total length (cm)*   | 32.6±2.6 (25.4-39.2)     | 31.9±2.4 (21.8-36.4)      |
| Condition factor (g cm⁻³)* | 0.010±0.001 (0.007-0.011)   | 0.009±0.001 (0.006-0.012) |
| Hepatosomatic index  | 1.0±0.4 (1.0-3.0)        | 1.1±0.3 (1.0-2.0)         |
| Gonadosomatic index* | 1.1±1.5 (0.0-8.0)        | 5.6±3.6 (0.0-18.0)        |
| Sex ratio (male/female/immature) | 11/24/5                  | 5/35/2                    |
| **Parasite Data**    |                         |                           |
| Prevalence (%) (number of infected hosts) | 85.0 (34)               | 83.3 (35)                 |
| Intensity *          | 21.7±20.2 (1-87)        | 16.4±40.9 (1-245)         |

Molecular characterization of anisakids

This procedure follows closely the one described in Kuhn et al. (2011). Eighteen and 20 anisakids (*Anisakis* sp. and *Hysterothylacium* sp.) from *S. colias* and *S. scombrus*, respectively, were analysed molecularly. DNA extraction was done for each larva, cut into pieces, using a genomic DNA extraction kit (peqGOLD MicroSpin Tissue, PEQLAB Biotechnology GmbH, Germany) and following the instructions of the manufacturer. The region of the rDNA included the ITS-1, 5.8S, ITS-2 and flanking sequences (=ITS+), and was amplified using the primers Forward TK1 (5’-GGC-AAA-AGT-CGT-AA-GAG-GT-3’) and Reverse NC2 (5’T-TA-TGT-TCT-TCT-CCG-CCT-3’). PCR reaction (50 µl) included 25 µl Master Mix (18.75 µl ddH₂O, 5 µl reaction buffer Y, 1 µl dNTP, 0.25 µl Taq-DNA-polymerase), 14 µl ddH₂O, 3 µl of each primer and 5 µl of genomic DNA. Each PCR reaction was performed in a PCR thermocycler (GeneTouch, Biozym scientific, Germany) under the following conditions: an initial denaturation at 95°C for 1 min, 40 cycles of 94°C for 45 sec (denaturation), 55°C for 45 sec (annealing), 72°C for 45 sec (extension), and a final extension at 72°C for 10 min. Samples with no DNA were included for negative control. PCR products were checked on 1% agarose gel that ran for 1 h 15 min at 80 V provided by an electrophoresis power supply (EV 245, Consort, Belgium) inside an electrophoresis system (MSMIDI, MULTISUB, Biozym, Germany). The gel was then analysed with a BiodocAnalyse (Biometra, AnalytikJena, Germany) associated with a photographic camera (Canon EOS 1100D) and BioDoc Analyse software version 2.66.3.22 (Biometra, AnalytikJena, Germany). A GelRed 100 bp DNA ladder marker (peqGold Leader Plus, PEQLAB Biotechnologie GmbH, Germany) was used to estimate the size of the PCR products. The agarose gel products were afterwards purified with a Cycle-Pure Kit (peqGold, PEQLAB Biotechnology GmbH, Germany).

Twenty µl of each DNA sample and 2.5 µl of each primer per sample was sent to the laboratory (GATC Biotec AG, European Custom Sequencing Centre, Cologne, Germany) for sequencing. In total, 38 worms were sequenced. The species were identified by comparison with sequences previously deposited in GenBank, using the BLASTn algorithm and the BioEdit software version 7.1.3.0 (Hall 1999) to previously aligned sequences forward and reverse provided by the sequence laboratory.

Infection levels of anisakids

Prevalence and intensity [mean±SD (minimum-maximum)] were calculated for each identified taxon according to Bush et al. (1997) and considering each of the two species of host. Binary Pearson correlations among anisakid intensity and host total weight, total length, condition factor, hepatosomatic index and gonadosomatic index were conducted. Moreover, the anisakid intensities were analysed in smaller versus larger fish for host total weight, total length, condition factor, hepatosomatic index, gonadosomatic index and sex (male versus female), using the Mann-Whitney’s U test. In the later analysis, the two classes of fish were separated by the median value for each parameter. In the case of detecting significant differences, the anisakid intensities [mean±SD (minimum-maximum)] for the smaller and bigger hosts were determined. Non-parametric tests were chosen for the statistical analysis because the study of the normality using the Kolmogorov-Smirnov’s test for some variables, such as anisakid intensity, showed that they did not follow a normal distribution (needed in parametric tests), as for instance.
Occurrence of *A. simplex* s.s. and *A. pegreffii* was compared between the two species of host using the chi-squared test. All statistical analyses were conducted in SPSS for Windows, version 23 (level of significance: *P*<0.05).

## RESULTS

### Anisakid infection levels

Both Atlantic mackerels recorded very high infection levels—a prevalence of anisakids of 85.0% and 83.3% for *S. colias* and *S. scombrus*, respectively. In total, 1312 anisakid worms were sampled from both fish, 737 from *S. colias* and 575 from *S. scombrus*. Mean intensity was higher for *S. colias*, with 21.7 worms/host, than for *S. scombrus*, with the 16.4 worms/host. These infection values were recorded for worms mainly found around the viscera, because the muscular tissue was found infected only once, with a single anisakid recorded in *S. scombrus* (prevalence of 2.4%) and none in *S. colias* (prevalence of 0%).

Binary correlations between anisakid intensity and host total weight, total length, condition factor, hepatosomatic index and gonadosomatic index were found to lack statistical significance.

The comparison of anisakid intensities among bigger and smaller hosts, separated by the median value, for host total weight, total length, condition factor, hepatosomatic index, gonadosomatic index and sex (males versus females) were in most cases non-significant, with probability values higher than 0.306, except for the gonadosomatic index, which was significant and recorded a probability value of 0.001. The intensities [mean±SD (range)] for the immature and mature hosts, according to the gonadosomatic index weight, were 6.0±8.6 (1-34) and 27.5±56.8 (2-245) worms per fish, respectively.

### Anisakid molecular identification

Among the 36 *Anisakis* worms sequenced, several sequences were deposited in GenBank (accession numbers KF923929 and KF923930 for *A. simplex* (s.s.) and KF923927 for *A. pegreffii* from *S. scombrus*; and KF923928 for *A. simplex* (s.s.), and KF914636 and KF923926 for *A. pegreffii* from *S. colias*). The distribution for each *Anisakis* species for both fish hosts is given in Table 2. *A. simplex* (s.s.) was significantly more frequent in *S. scombrus* than in *S. colias*, whereas the opposite trend was reported for *A. pegreffii* (*χ²*=9.03, *P*<0.01).

*Hysterothylacium aduncum* specimens were also occasionally recorded, and two worms were sequenced, one worm per fish host species (GenBank accession numbers KF923932 from *S. scombrus* and KF923931 from *S. colias*, both showing 99% similarity to the *H. aduncum* sequences HM598666.1 and JQ934883.1 (Smrzlić et al. 2012)).

### DISCUSSION

In this study, infection levels for anisakids were evaluated considering two species of Atlantic mackerel, *S. colias* and *S. scombrus*, sampled at the continental Portuguese coast. The high infection levels (83%–85% prevalence) recorded for both fish are greater than the 10% reported for *S. scombrus* off continental Portugal by Rego et al. (1985), and the 69.5% reported for *S. colias* off the Madeira Islands, Portugal, by Costa et al. (2003). However, they are lower than the 100% reported for *S. colias* off the Azores Islands, Portugal, by Shukglalter (2004). High prevalence values were also recorded in other geographic regions: 87% for *S. colias* in El Rincon, Argentina (Cremonte and Sardella 1997) and 92% for *S. scombrus* and 100% for *S. colias* in the Adriatic Sea off Croatia (Mladineo 2003, Mladineo and Poljak 2014). In contrast, in Moroccan waters, prevalence of anisakids in *S. colias* varied from 57% to 67.9% (Abattouy et al. 2011). High prevalence values were also recorded for anisakids of other fish species from the Portuguese mainland, such as the blue whiting (*Micromesistius poutassou*, 77.7%), the black scabbardfish (*Aphanopus carbo*, 100%) (Cruz et al. 2007, 2009) and the blackspot seabream (*Pagellus bogaraveo*, 100%) (Hermida et al. 2012). Moreover, Costa et al. (2004) also reported a high prevalence (89.6%) for the blackspot seabream off Madeira Islands, Portugal.

In our samples, not only the overall prevalence but also the mean intensity of anisakids was high, with 21.7 worms per host for *S. colias* and 16.4 worms per host for *S. scombrus*. These values were similar to the ones recorded from the Azores Islands (Hyeres Bank), with 17.1 worms per host for *S. colias* (Shukglalter 2004).

The correlation analysis of anisakid intensity and host total weight, total length, condition factor, hepatosomatic index and gonadosomatic index for *S. colias* and *S. scombrus* was found not to be significant. Moreover, the comparison analysis of anisakid intensity among smaller and bigger fish according to host total weight, total length, condition factor, hepatosomatic index, gonadosomatic index and sex (males versus females) showed mostly the same behaviour: the results were non-significant, except for the gonadosomatic index in *S. scombrus*. In the latter, more mature fish, with higher index values, recorded significantly higher intensities of anisakids than fish with lighter gonads, with mean values of 6.0 and 27.5, respectively. These findings can be interpreted as a conspicuous high preference for the first intermediate host by the more mature hosts. The lack of significant values for correlation between intensity of anisakids and host data was also recorded by Mladineo and Poljak (2014) for *S. colias* and *Sardina pilchardus* in the Adriatic Sea, and by Costa et al. (2004) for *Pagellus bogaraveo* off Madeira Island. By contrast, Cruz et al. (2009)
recorded a significant increase in anisakid intensity according to the host length for Aphanopus carbo off Sesimbra (mainland) and Madeira Islands, Portugal. Additionally, Chou et al. (2011) found significantly higher intensity of Anisakis larvae in larger and older spotted mackerel (Scomber australasicus) off the Taiwanese coast. Furthermore, Mladineo and Poljak (2014) recorded significant values for host length and abundance of Anisakis from anchovies (Engraulis encrasicolus), European hake (Merluccius merluccius) and whiting (Merlangius merlangus) in the Adriatic Sea. The correlation significance between either intensity or abundance of Anisakis and host length, seems to be dependent on the fish species, and for S. colias the present work is at least the second time that it has been measured (Mladineo and Poljak 2014), but with no significance.

In our study, the edible portion of the fishes, the muscle, recorded low or absent infection values, with 2.4% for S. colias and 0% for S. colias. Nevertheless, a potential risk of infection should not be discarded, because anisakid larvae can migrate from the viscera to the muscle after death of the host (Smith and Wootten 1975, Hauck 1977). These results suggest that fish caught from Portuguese northern Atlantic waters are highly susceptible to carry infections with anisakid nematodes and thus represent a potential human health problem. Taking into account these infection values, we advise that Atlantic mackerels caught off Portugal be ingested with care, and only after being properly frozen or cooked. The European Union (2011) recommends that for the safe consumption of fish products, raw or undercooked, a previous period of freezing and storage at a core temperature of –20°C or below for not less than 24 h, or of –35°C or below for not less than 15 hours should be applied. In addition, the recommendation of fish evisceration and low temperatures on board just after the catch should be followed more closely. These procedures will avoid as much as possible the migration of the worm to the muscle after the host dies.

The present study identifies for the first time the anisakid species found in mainland Portuguese Atlantic mackerels, S. colias, and recognizes the presence of mixed infections with both A. simplex s.s. and A. pegreffii in the two scombrid fish species. For S. colias off Madeira Island, Pontes et al. (2005) recorded six different Anisakis species for S. colias, namely A. simplex s.s., A. pegreffii, A. nascetti, A. typica, A. ziphidium and A. physeteris. The detection of A. simplex (s.s.) and A. pegreffii mixed infections have also been recorded for S. colias in Galicia from the northwestern coast of Spain (Abollo et al. 2001), and for S. colias and S. colias in the Alboran Sea off the southern coast of Spain (Abollo et al. 2003). These findings corroborate the statement that the Portuguese and Spanish coasts are a sympatric area for both parasite species (Abollo et al. 2001, Marques et al. 2006).

Taking into account the distribution of the different Anisakis species in our fish sample, we further hypothesize that this pattern may be related to differences in the anisakid life cycle hosts, since the two fish mainly occur at different depths. A. pegreffii larvae are probably found in deeper waters, as are their main first host, than A. simplex (s.s.) larvae in less deeper waters, because A. pegreffii larvae were more frequent in S. colias (a deeper fish living at 50-300 m) than in S. colias (living at 0-200 m depth). However, this subject is controversial. According to Mattiucci et al. (1997), A. simplex (s.s.) is found mainly in benthic or demersal fishes, while A. pegreffii is found mainly in pelagic fishes, based on the marine mammal final host distribution. Abollo et al. (2001) confirmed the occurrence of A. simplex (s.s.) in benthic and demersal fishes but could not confirm the occurrence of A. pegreffii in pelagic fishes, having analysed a range of different fishes and cephalopods. Kuhn et al. (2013) found that both Anisakis species have similar proportions of fish hosts that live in pelagic, benthopelagic and demersal environments. However, after a second analysis of this author’s data, we may see that A. pegreffii occurs in a higher number of reef-associated fish species than A. simplex (s.s.), thus supporting our hypothesis. Also, the high abundance of A. pegreffii in Trachurus trachurus (Abattouy et al. 2013), a benthopelagic fish reaching depths of 0 to 1050 m (Lloris and Moreno 1995), supports our hypotheses. The geographic distribution of a parasite will be the conjunction of the distributions of its different hosts belonging to its life cycle. And a parasite with high host specificity will have a smaller distribution, probably limited to that of its hosts, than a non-specific parasite, which is expected to have a broad distribution.

We are aware that further data on the distribution within the Anisakis simplex complex at the invertebrate host level is needed in order to prove our hypothesis. Smith (1983) proposed that euphausiids were the major intermediate host of A. simplex s.l., in the northeast Atlantic and northern North Sea, and perhaps universally. Recently, Gregori et al. (2015) detected the occurrence of larvae of A. simplex (s.s.) and A. pegreffii in the euphausiids Nyctiphanes couchii, justifying the co-occurrence of both species in the same fish species, and its sympathy in the northeast Atlantic. However, other invertebrate species were also indicated as host candidates for Anisakis species, such as copepods (eg. Acartia tonsa, and Oitona similis) (Koie 2001). The low infection levels recorded for these first intermediate hosts to date in the ocean do not allow us to fully understand what species are more important for the life cycle of each Anisakis species, but further research studies will certainly solve this problem in the short term.

According to earlier studies, the two recorded Anisakis species have a different potential zoonotic level in humans: A. simplex (s.s.) is more prone to migrate into the muscle than the other species, and also withstands better the gut juices of the human stomach, with high survival rates (Arizono et al. 2012). These differences in parasite behaviour explain why more human infections have been reported due to A. simplex (s.s.) than to A. pegreffii in Japan, where both species co-occur and the ingestion of raw fish is particularly common (Umehara et al. 2007). Moreover, the average number

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of Anisakis muscle larvae in Scomber japonicus can be 12 times higher for A. simplex s.s. than for A. pegreffii. (Suzuki et al. 2010). Taking into account that our Atlantic mackerels have significantly different infections with these two Anisakis species and likewise a similar anisakid load, we may conclude that it is less safe to eat S. scombrus, since it harbours more A. simplex (s.s.) than S. colias, unless it is appropriately frozen or well cooked.

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