Anisakis infection in allis shad, *Alosa alosa* (Linnaeus, 1758), and twaite shad, *Alosa fallax* (Lacépède, 1803) from Western Iberian Peninsula Rivers: zoonotic and ecological implications

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Abstract

Spawning individuals of allis shad, *Alosa alosa* (Linnaeus, 1758), and twaite shad, *Alosa fallax* (Lacépède, 1803) were sampled from three rivers on the Atlantic coast of the Iberian Peninsula (Ulla, Minho, Mondego) during 2008 to 2013 to assess the presence of the zoonotic marine parasite *Anisakis* spp. larvae. The results revealed that both shad species were infected by third-larval stage *Anisakis simplex* s.s. and *Anisakis pegreffii*. The latter is reported in mixed infections in both shad species of Western Iberian Peninsula for the first time. In *Alosa alosa* the prevalence of *Anisakis* infection can reach 100%, while in *Alosa fallax* prevalence was up to 83%. Infected individuals of the former species also often contain much higher number of parasites in theirs internal organs and flesh: from 1 to 1138 *Anisakis* spp. larvae as compared to 1 to 121 larvae, respectively. In general, numbers of *A. pegreffii* were higher than those of *A. simplex* s.s. Our results suggest that in the marine environment of the Western Iberian Peninsula both anadromous shad species act as paratenic hosts for *A. simplex* s.s. and *A. pegreffii*, thus widening the distribution of the infective nematode larvae from the marine to the freshwater ecosystem. This finding is of great epidemiological relevance for wildlife managers and consumers, considering the zoonotic and gastro-allergic threats posed of these parasites.

Key words

Alosa, Anisakis, Iberian Peninsula, anadromous, freshwater, gastro-allergic.

1. Introduction

Allis shad, *Alosa alosa* (Linnaeus, 1758), and twaite shad, *Alosa fallax* (Lacépède, 1803), are anadromous members of the family Clupeidae. In their marine phase they live mainly in coastal waters and have a pelagic lifestyle; they migrate to rivers for spawning (Aprahamian et al. 2003; Baglinière et al. 2003). Historically, their distributions along the eastern Atlantic coast extended from Iceland and Norway in the north to Morocco in the south (Aprahamian et al. 2003). In addition, *A. fallax* is found throughout the Mediterranean Sea although rare in the Marmara and Black sea, whilst *A. alosa* formerly occurred in the western Mediterranean (Ceyhan et al. 2012; Faria et al. 2012).

Both species are protected and fishing regulated by Spanish[1] and Portuguese[2] legislation. In addition, *A. alosa* is classified as "endangered" and *A. fallax* as "vulnerable" in the Red Book of the Portuguese Vertebrates (Cabral et al. 2006) and both species are considered as "vulnerable" by some authors in Spain (Doadrio et al. 2011). In Galicia (NW of Spain), *A. alosa* is classified as "vulnerable" in the Galician List of Threatened Species (DOG 2007), and both shad species are considered as "endangered" in Galician Rivers by some authors (Solórzano 2004).

Currently, the international section of River Minho (ISRM), along the border between Spain and northern Portugal, holds what seems to be the only stable *A. alosa* stock in this region (Mota et al. in press). It suffered a dramatic drop in annual catches, by about 90%, after the first half of the 20th Century but numbers subsequently stabilized (Mota and Antunes 2011; Mota et al. in press). *A. fallax* occurs in the River Ulla (Cobo et al. 2010; Nachón et al. 2013; Silva et al. 2013), and also inhabits the River Minho (Migranet 2012). Migration of *A. alosa* occurs mainly from March to June in the River Minho (Mota and Antunes 2011; Mota et al. in press). The upstream reproductive migration of *A. fallax* seems to take place between March and July in the River Ulla

[1] DOG 2012, BOPDEPO 2013

[2] Decree-Law Nº. 140/99 of April 24th, Decree-Law Nº. 316/89 of September 22nd, Law Nº. 2097 of June 6th 1959, Regulatory Decree Nº. 43/87 of July 17th, Regulatory Decree Nº. 7/2000 of May 30th and Decree-Law Nº. 8/2008 of April 9th

(Nachón et al. 2013) and between April and June in the River Minho (M. Mota unpublished data).

These anadromous species, especially *A. alosa*, have considerable ecological, sociocultural and economic importance in Galicia, in the vicinity of the River Minho, and, especially, in Portugal (Pereira et al. 2013) as for the whole of their distribution range (Baglinière 2000). Commercial fishing is permitted in the lower ISRM (BOPDEPO 2013). Spanish vessels caught around 2000 *A. alosa* per year with trammel nets in 2010/11 and 2011/12 (data provided by Comandancia Naval de Tui). Portuguese catches are similar (Mota and Antunes 2011). The socioeconomic importance of *A. alosa* is reflected in their high commercial value, which can range between 10 €/kg to 16 €/kg, depending on the year. Spanish *A. fallax* catches were less than 200 fish per year in 2010/11 and 2011/12 (data provided by Comandancia Naval de Tui). Sport fishing is permitted in the River Minho for both species (BOPDEPO 2013) and also for *A. fallax* in the River Ulla (DOG 2012).

Nematodes of the genus *Anisakis* are marine parasites belonging to the family Anisakidae, for which cetaceans serve as the main final hosts. It is considered that euphausiaceans and copepods act as intermediate hosts and the anisakids also use a huge variety of fish and cephalopods as paratenic or transport hosts (Mattiucci and Nascettii 2008). Humans may become an accidental host when they eat at least one live larva of *Anisakis* spp. from raw or undercooked seafood products. This ingestion can cause clinical pathology, namely anisakiasis or gastroallergic problems associated to thermostable allergens from third-stage larvae. The anisakids with most zoonotic relevance are *Anisakis simplex* s.s. (Rudolphi, 1809) and *Anisakis pegreffii* (Campana-Rouget and Biocca, 1955), which both belong to *Anisakis simplex* complex, and which are the etiological agents responsible for an increasing number of clinical cases worldwide (Arizono et al. 2012; Mattiucci et al. 2013; Juric et al. 2013). These two *Anisakis* species seem to have different host, life cycle and distribution preferences within European waters, although they are

known to co-infect fish hosts along the Spanish and Portuguese Atlantic coast and in the Alboran Sea (Mattiucci and Nascetti 2006; 2008).

In Galicia, *A. alosa* and *A. fallax* are usually prepared fried in thin slices or baked. In Portugal several recipes exist, especially for *A. alosa*, including its roe, which is considered to be a delicacy, but in all cases the fish is well-cooked (Pereira et al. 2013; C. Antunes and M. Mota, *pers. obs.*). However, consumers may still experience a reaction to thermostable parasite allergens, if present.

The present paper provides an overview of the epidemiology of *Anisakis* spp. in both shad species in this area, with the aim of identifying the different species present and obtaining quantitative descriptors of parasite populations. We highlight the zoonotic risk for wildlife and consumers, and discuss the ecological implications of our findings on these two vulnerable shad species.

2. Materials and methods

2.1. Sampling

Several sample batches of shads (Table 1) were caught by experimental or professional fishing (trammel net) or by sport fishing in Minho, Ulla and Mondego rivers (Fig. 1), covering March to August from 2008 to 2013 to include whole migration season. For more detailed descriptions of the sampling sites see Mota et al. (in press), Mota and Antunes (2011) and Nachón et al. (2013).

2.2. Necropsy and visual inspection

Data on total weight (TW), total length (TL) and sex were recorded for each specimen. A longitudinal section was performed from the cloaca to the operculum and then upwards to expose internal organs. Internal organs were removed and macroscopic observation was carried out to detect free *Anisakis* larvae around the peritoneum in the empty visceral cavity. Internal organs were then inspected for presence of *Anisakis* larvae or frozen for subsequent visual

inspection. Most stomachs were separated from viscera and visually inspected, in order to identify the preferences of nematodes for tissue location. Next, some samples of the visceral cavity (without stomach) and fish flesh were subjected to further enzymatic digestion, in order to confirm the visual inspection. The branchial region of most shads belonging to batch 2, 6 and 7 (Table 1) was dissected and gill arches were extracted and examined for *Anisakis* larvae.

All nematodes found by macroscopic observation or enzymatic digestion were separated and conserved in ethanol 70%. Then, every anisakid larva from each tube was individually examined and identified at genus level under a stereomicroscope. Only anisakid nematodes belonging to the genus *Anisakis* spp. were included in the present study. Finally, a random selection of parasite samples from individual shads and organs was stored for molecular identification of *Anisakis* species.

2.3. Artificial enzymatic digestion

The artificial digestion of the flesh and visceral cavity of shads was carried out on the basis of an optimized artificial digestion protocol (Llarena-Reino et al. 2013a). The flesh was digested at 37-40°C during approximately 3-4 hours (3 hours for visceral cavity material) in an ACM-11806 Magnetic Stirrer Multiplate, using a weight/volume pepsin ratio of 1:20, understanding that ratio as 20 ml of a 0.5% pepsin solution in HCl 0.063M (pH 1.5) for 1 g of flesh. The digestion solution was decanted through a sieve and the residues of digestion and nematodes were inspected under stereomicroscope. All *Anisakis* spp. found were placed in individual tubes with ethanol 70%.

2.4. Molecular analysis

Genomic DNA from 72 *Anisakis* spp. larvae was individually isolated using MACHEREY-NAGEL NucleoSpin®Tissue kit following manufacturer-recommended protocols. The entire ITS (ITS1, 5.8S rDNA gene and ITS2) was amplified using the forward primer NC5 (5´- GTA

GGT GAA CCT GCG GAA GGA TCA TT-3´) and reverse primer NC2 (5´- TTA GTT TCT TTT CCT CCG CT-3´). PCR reactions were carried out in a total volume of 25µl containing 100 ng of genomic DNA, 10 µM of each primer, 2.5 µl of 10x buffer, 0.5 µl of dNTPs and 5 U/µl of Taq DNA polymerase (From Thermus Aquaticus BM, recombinant, Roche). PCR cycling parameters included denaturation at 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 sec., annealing at 55º C for 30 sec., and extension at 72º C for 1min. 15sec., and a final extension at 72º C for 7 min. PCR products were purified using illustra ExoStar 1-Step following manufacturer recommended protocols, with some modifications. We added 4 µl of reactive illustra ExoStar 1-Step and incubated the mix for 15 min. at 37º C. For inactive the reactive added we incubate the mix 20 min. at 80º C. Samples with DNA concentration in clean reaction of 20 ng/µl were sequenced by SECUGEN® (Madrid). All sequences were subjected to a homology search through Basic Local Alignment Search Tool (BLAST) searches in the National Center for Biotechnology Information (NCBI) database.

2.5. Quantitative descriptors and statistical analysis

Quantitative descriptors of parasite populations found in shads, such as prevalence, mean abundance and mean intensity were calculated as described in Bush et al. (1997).

Factors affecting the parasite burden of both shad species were investigated using a generalized additive modelling (GAM) framework as implemented in Brodgar 2.7.4 (http://www.brodgar.com/). The response variable was the number of *Anisakis* spp. larvae found in the visceral cavity (including stomach) of fish, using visual methods. The explanatory variables considered for the model selection process were: TL, TW, sex, condition factor (K [$K = 100 \times (TW/TL^3)$]), river, river section, year, day of the year (expressed as a fraction of 365 days), and observer. All data series were explored for outliers, collinearity, heterogeneity of variance and interactions between variables, and to visualize the relationships between response and explanatory variables, following the protocol proposed by Zuur et al. (2010).

The sampling date was expressed as a fraction of the calendar year (yearfrac). Moreover, yearfrac was correlated with K, hence in order to remove the season (yearfrac) effect from latter variable, it was "de-seasonalised" by regressing against yearfrac (treating yearfrac as a smoother [k=4]). Thus in the models, K is substituted by resulting residuals, becoming "res K". Note that K is derived from TL and TW and therefore was not included in the same models as TW. The variables river section and observer could not be included in the same model since, for some samples, the two variables are confounded.

For the *A. alosa* dataset, *Anisakis* numbers approximated to a Gaussian distribution after cubic-root transformation and we therefore used a Gaussian GAM with identity link function. For the *A. fallax* the data were more strongly skewed with an excess of zeroes but a quasi-Poisson GAM (with log link) provided a satisfactory solution. In both cases, forwards selection was applied to identify the best models. For Gaussian GAMs, the optimum model was the one with the lowest value for the Akaike Information Criterion (AIC, Akaike 1974) provided that deviance explained was reasonably high and individual explanatory variables had significant effects. For the quasi-Poisson models, the AIC is not available and selection was based on the latter two criteria. Smoothers obtained by cross-validation for effects of TL, yearfrac and res K on *Anisakis* abundance in *A. fallax* were unrealistically complex and models were refitted after setting a maximum k value of 4. Final models were checked for robustness to addition of further explanatory variables as well as for problems such as influential data points or trends in residuals.

3. Results

3.1 Parasite inspection

The visceral cavity of *A. alosa* specimens was frequently clearly infected, as seen by visual inspection. The larvae were found rolled or free on the exterior surface of the internal organs, especially the pyloric caeca, connective tissue, fat and gonads. Moreover, they were also found

on the surface of intestine, liver and spleen. In addition, visual inspection of the flesh revealed the presence of marks probably caused by *Anisakis* larvae. Accumulation of *Anisakis* larvae was usually observed at the posterior end of the terminal blind sac of the stomach. When the accumulation was clearly evident, the stomach wall appeared broken, presumably due to the parasites' migration from the stomach to visceral cavity (Fig. 2). No *Anisakis* were observed in the gills.

On the contrary, based on visual inspection, the visceral cavity of *A. fallax* generally seemed to be lightly infected. Nevertheless, occasionally, several internal organs appeared clearly infected (Fig. 3). No *Anisakis* larvae were detected visually in the flesh or gills.

Visual inspection indicated a prevalence of *Anisakis* of 100% for *A. alosa*, but only 35% for *A. fallax*.

3.2 Genetic identification

All isolated anisakid larvae were initially examined under the stereomicroscope enabling identification to the genus level. Furthermore, several *Anisakis* spp. larvae from every batch were subjected to molecular diagnosis. According to the ITS amplified regions of 750 bp and searches for sequence homology (Blast values of 100%), the nematode species identified from both shads belong to *A. simplex* s.s. and *A. pegreffii*. Parasite sequences were deposited in the Gen Bank (Accession numbers KP857639-KP857649). Based on the molecular work, *A. pegreffii* is more numerous than *A. simplex* s.s. in the samples, although there was some variation between rivers (Table 2).

Both species of *Anisakis* have been diagnosed in the flesh of *A. alosa* from the River Minho. Four larvae were tested genetically, three of which were *A. simplex* s.s. and one *A. pegreffii*. The single larva found in the flesh of *A. fallax* from the River Ulla was genetically identified as *A. simplex* s.s.

3.3 Infection data

The quantitative descriptors of *Anisakis* spp. larvae of both shad species from all sampling batches are shown in Table 3. *Alosa alosa* showed higher values than *A. fallax* for every quantitative descriptor of *Anisakis* infection. Thus, following visual and enzymatic digestive detection methods (i.e. in batches 2 and 7), *A. alosa* from the River Minho showed total mean abundance and intensity parameters hundreds of times higher than *A. fallax* from the same river, although it should be noted that the species were sampled in different years (i.e., 2013 and 2012, respectively). In relation to infection in the flesh, *A. alosa* were clearly infected with moderate values of *Anisakis* abundance up to a maximum of 13 larvae per fish. On the other hand, *Anisakis* infection of *A. fallax* flesh was almost absent, with only a single larva found in one fish of 73 inspected following enzymatic digestive methods. Notwithstanding the foregoing, the vast majority of larvae were located in the visceral cavity (including stomach) for both shad species. In addition, *A. alosa* usually showed an aggregation of *Anisakis* larvae in the visceral cavity, located at the posterior end of the terminal blind sac of the stomach (Fig. 2). Specimens from River Minho (batch 2) showed an overall mean of 313.83 larvae per aggregation (range 8 to 884 larvae).

3.4 Statistical modelling

Both final models were satisfactory in terms of an absence of highly influential data points and of trends in residuals. Results from the GAMs indicated that the numbers of parasites in *A. alosa* were significantly related to TL ($p < 0.0001$), yearfrac ($p = 0.0001$), residual K ($p = 0.0006$), year ($p < 0.0001$) and river ($p = 0.001$). The years with the lowest numbers of parasites were 2010 and 2011, whilst 2012 and 2013 had highest values. Moreover, samples from the River Mondego showed fewer parasites than those from the River Minho. The model explained 71.5% of deviance. Smoothers presented in Figure 4 suggest that the number of parasites in the visceral cavity of *A. alosa* increased with TL and residual K. A significant effect was also shown for

yearfrac, with a decreasing number of parasites until April, followed by a rise until the end of May.

The numbers of parasites in *A. fallax* were significantly related to TL ($p < 0.0001$), yearfrac ($p = 0.0003$), residual K ($p = 0.0005$), sex ($p < 0.0007$) and river ($p < 0.0001$). The samples with fewest parasites were those from the River Ulla, whilst males showed more parasites than females. The model explained 35.8% of deviance. As for the *A. alosa* model, the smoothers presented in Figure 4 suggest that the number of parasites in the visceral cavity of *A. fallax* increased with TL and residual K. Again the number of parasites decreased from the end of March until the end of April followed by a rise until the middle of July.

4. Discussion

The marine parasitic nematode *A. simplex* is well reported in various *Alosa* spp. (Landry et al. 1992; Hogans et al. 1993; Shields et al. 2002). However, epidemiological studies of parasites in European shads remain scarce. Knezevic et al. (1978) and Quignard and Douchement (1991) reported *Anisakis* sp. larvae from *A. fallax nilotica* and *A. fallax*, respectively. Moravec (2001) reported *A. simplex* in a specimen of *A. alosa* from the River Elbe. Rokicki et al. (2009) reviewed the presence of *A. simplex* in *A. fallax* from Baltic Sea. Recently, Mota et al. (in press) presented the first report of *A. pegreffii* in *A. alosa* from the River Minho.

Where are shads infected by Anisakis *nematodes?*

Several previous studies have shown the usefulness of anisakid nematodes as biological tags for fish stock characterization in European waters (MacKenzie 2002; Mattiucci et al. 2008; Kuhn et al. 2011). In relation to this, the presence of a mixed infection of *A. pegreffii* and *A. simplex* s.s. in *A. alosa* and *A. fallax* of the western Iberian Peninsula is in agreement with previous epidemiological information for other fish species studied in Western Iberian marine waters. Along the eastern coast of the Atlantic Ocean, the distribution of *A. simplex* s.s. seems to have a

southern limit around the Strait of Gibraltar. *A. pegreffii* is the main species of *Anisakis* in the Mediterranean and it is also widely distributed along East Atlantic Ocean down to the Antarctic Peninsula (Mattiucci and Nascetti 2008; Khun et al. 2011). The West Iberian Peninsula coast represents an oceanic area where several fish species have been found with such mixed infections, as were two toothed whale species belonging to the family Delphinidae, short-beaked common dolphin, *Delphinus delphis*, (Linnaeus, 1758), and long-finned pilot whale, *Globicephala melas*, (Traill, 1809) in NW Iberian Peninsula waters (Abollo et al. 2001; 2003; Mattiucci et al. 2004; 2007; 2014; Mattiucci and Nascetti 2008; Hermida et al. 2012).

Within this area of sympatry, analysis of mixed infections has revealed different relative proportions of *Anisakis* species depending on the geographical distribution of the host fish species (Mattiucci et al. 2004; 2007; 2008; Mattiucci and Nascettii 2008; Hermida et al. 2012). The fact that shad from Galician and Portuguese Rivers had a higher proportion of *A. pegreffii* than *A. simplex* s.s. might suggest three different hypotheses:

Firstly, previous parasitological studies carried out along the Western Iberia Coast have showed an increasing relative proportion of *A. pegreffii* (and the opposite for *A. simplex* s.s.) from North (Galicia) to South (coasts of South Portugal) in horse mackerel, *Trachurus trachurus* (Linnaeus, 1758) (Mattiucci et al. 2008). Abollo et al. (2003) found the highest prevalence of *A. simplex* s.s. in the North of the Iberian Peninsula, decreasing towards the south, and the opposite tendency for *A. pegreffii*, which had the highest prevalence in the Alboran Sea and the lowest in the Cantabrian Sea. Other studies have shown a higher relative abundance of *A. pegreffii* in blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), from Portuguese waters of the Iberian Coast (Hermida et al. 2012). Hybrids of *A. simplex* s.s. and *A. pegreffii* can be found in some fish species in this area and, occasionally, other *Anisakis* spp. are found (Abollo et al. 2003; Marques et al. 2006; Sequeira et al. 2010; Hermida et al. 2012). Generally, studies carried out in Galician waters have shown mixed infections with a higher proportion of *A. simplex* s.s. in

blue whiting, *Micromessistius poutassou* (Risso, 1827); European hake, *Merluccius merluccius* (Linnaeus, 1758) and *T. trachurus* (Abollo et al. 2003; Mattiucci et al. 2004; 2008). In addition, single infections of *A. simplex* s.s. were confirmed genetically in four cephalopod and seven fish species, whilst mixed infections were confirmed in seven fish species (Abollo et al. 2001). Interestingly, sea lamprey, *Petromyzon marinus* (Linnaeus, 1758) from the River Ulla and River Tea (tributary of the River Minho) showed a high prevalence of *A. simplex* s.s. (Bao et al. 2013). In addition, a single larva of *A. pegreffii* was found in one *P. marinus* from the River Ulla (M. Bao unpubl. data). Hence, we suggest that both shad species migrate southward temporarily to feeding grounds off central or southern Portugal thus acquiring a relatively high proportion of *A. pegreffii*.

A second possibility is that there is immigration of shad from Mediterranean or NW African stocks. This explanation seems fairly unlikely due to previous suggested homing behaviour (Alexandrino 1996; Sabatié et al. 2000), the existence of three different haplogroups throughout the Atlantic basin and the geographic distribution of genetic diversity within both shad species, which suggests the existence of a strong but permeable barrier among Atlantic and Mediterranean populations (Jolly et al. 2012; Faria et al. 2012). Moreover, both Moroccan shad populations are considered almost extinct (Sabatié and Baglinière 2001).

Thirdly, it is possible that the predominance of *A. pegreffii* is extending northwards. In relation to this, a recent parasitological study carried out on *M. poutassou* from ICES fishing area Div. VIIIc found a slightly higher proportion of *A. pegreffii* (6 larvae of *A. pegreffii* and 4 larvae of *A. simplex* s.s.) (Llarena-Reino et al. 2013b). However, to date, as far as we known, other paratenic fish species from coastal waters of NW Iberian Peninsula do not show a higher relative proportion of *A. pegreffii.* Further studies will be needed to determine which, if any, of these explanations is correct.

How do shads acquire Anisakis *parasites?*

Alosa alosa is mainly a zooplanktophagous fish, the preferred prey of which are mainly Mysidacea, Euphausiacea (e.g. *Nycthipanes couchii* (Bell, 1853)) and copepods, and fish are secondary prey (Taverny and Elie 2001a; Aprahamian et al. 2003; Mota et al. in press). In contrast, *A. fallax* is essentially ichthyophagous (Assis et al. 1992; Taverny and Elie 2001a) and zooplankton (such as *N. couchii*) constitutes secondary prey (Taverny and Elie 2001a). The euphausiid *N. couchii* has been recently diagnosed as the intermediate host of both *A. pegreffii* and *A. simplex* s.s. in Galician waters (Gregori et al. in press). Thus, both shad species might gain their mixed infection of both *Anisakis* species by feeding on infected zooplankton (such as *N. couchii*) or other transport hosts during the marine trophic phase. Accumulation of anisakids by continuous reinfection through the diet is well-reported in several fish species (Mladineo and Poljak 2014 and references therein). Moreover, positive correlations of fish length and age with the number of larvae accumulated have been found in several fish species (Strømnes and Andersen 2003; Levsen and Lunestad 2010; Mladineo and Poljak 2014). Bearing in mind the latter findings and the fact that *Anisakis* infection numbers vary with fish species, fishing area and season (Mladineo et al. 2012 and references therein), it is possible that *A. alosa* present higher numbers of larvae than *A. fallax* because the former feeds intensively on zooplankton while the latter feeds mainly on small pelagic fish, such as sandsmelt, *Atherina boyeri* (Risso, 1810) (Nachón et al. 2013), which supposedly have a low *Anisakis* burden.

From an ecological point of view, it was suggested that transport hosts of *A. simplex* s.s. are mainly benthic or demersal, whilst those of *A. pegreffii* are mainly pelagic. Hosts with mixed infections, like shads, are meso- or benthopelagic (Mattiucci et al. 1997). Subsequently, it has been suggested that these parasites use pelagic and demersal food chains to complete their life cycles (Mattiucci and Nascetti 2008; Mattiucci et al. 2014). Both shad species seem to use pelagic and neritic environments, and also have schooling behaviour, although *A. fallax* seems to have a distribution pattern more dependent on estuarine environment, especially in younger individuals (Taverny and Elie 2001b). The ranges of depth distribution of both shad species,

recorded by observers on board the commercial fleet fishing over the continental shelf (generally >100m. depth) in NW Iberian Peninsula waters, seem to be in accordance with these results; nevertheless both species usually appear in the oceanic zone and in the epipelagic and mesopelagic environments. Bearing in mind that the observer data does not cover the coastal zone, it can be said that *A. alosa* occurs between 9 and 311 m (mean depth 174 m) while *A. fallax* occurs between 18 and 390 m (mean depth 148 m) (Data provided by Vigo IEO).

In the Sado estuary (Portugal), bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) predates *A. fallax* (Aprahamian et al. 2003 and references therein). Furthermore, Black Sea *T. truncatus* predates *Alosa* sp. (Gladilina and Gol´din 2014). In fact, *A. alosa* and other members of the subfamily Alosinae have been shown to respond to ultrasonic clicks from delphinids, which is consistent with a prey-predator relationship among these species (Wilson et al. 2011). Hence, shad are a suitable transport host for *A. simplex* s.s. and *A. pegreffii* in order to reach a suitable final host in marine or brackish environment of the Iberian Coast.

Statistical analysis

In both shad species, numbers of parasites were positively related to (the partial effects of) fish length and (seasonally adjusted) condition factor. In addition, numbers of parasites fell to a minimum in April, and then increased again to around the end of May (*A. alosa*) or middle of July (*A. fallax*), after which no trend could be discerned. There were also significant differences between rivers (both species), years (*A. alosa* only) and sexes (*A. fallax* only).

In part, these results are expected: Levsen and Lunestad (2010) found a highly significant effect of fish host size on total *Anisakis* larval abundance in another clupeid, Atlantic herring, *Clupea harengus* (Linnaeus, 1758), from Norwegian waters. Furthermore, *Anisakis* larvae accumulate with the increasing fish age and length in other fish species (Strømnes and Andersen 2003; Mladineo and Poljak 2014).

The seasonally adjusted condition factor also showed a positive correlation with parasite abundance. Despite the fact that parasites may be detrimental to their host (Rokicki et al. 2009), this positive relationship could arise simply because good condition and high parasite burden both reflect a high feeding rate (Mladineo and Poljak 2014).

The seasonal pattern in infestation is less easily explained. The spawning migration into freshwater has been reported to have effects on parasites of shads (Aprahamian et al. 2003) and, bearing in mind that the *Anisakis* larvae could not be acquired in such concentrations by shads in freshwater habitats, a progressive decrease in parasite burden over time spent in freshwater is plausible. Whilst *A. fallax* may feed during the spawning migration (Nachón et al. 2013), *A. alosa* do not feed while migrating (Mota et al. in press) and, in any case, *Anisakis* spp. are marine parasites. Thus the rise in average parasite burden from April to around the end of May (*A. alosa*) or middle of July (*A. fallax*) possibly indicates the later arrival of fish with higher parasite burdens. However, it is not obvious why this should occur and further research will be needed to confirm this trend and investigate the causes.

Risk assessment

The role of anadromous shad populations in the life cycle of *Anisakis* spp. from western Iberian Peninsula waters has important public health implications. *A. simplex* s.s. and *A. pegreffii* are the main zoonotic nematodes so far recognized as causing human anisakiasis and gastroallergic reactions (Arizono et al. 2012; Juric et al. 2013; Mattiucci et al. 2013). The European Food Safety Agency (EFSA) published a scientific opinion on risk assessment of parasites in fishery products (EFSA 2010). There, it was recognized that all wild-caught marine and freshwater fish are must be considered at risk of containing viable parasites of human health concern if these products are eaten raw or almost raw. Shad products are consumed fresh locally in the Iberian Peninsula and also in France (Elie et al. 2000), and are likely to be reasonably safe, due to the Portuguese and Galician cultural traditions of eating heavily-cooked food. Nevertheless, a

potential health human risk exists since allergic reactions could occur due to thermostable allergens even if no live larvae reach the consumer (Sharp and Lopata 2014; Baird et. al. 2014, Arcos et al. 2014). The recognition of several thermostable *Anisakis* antigens provoking allergic reactions, which can be highly aggressive and generate severe clinical manifestations, suggests that surveillance and epidemiological awareness should be encouraged. Apart from a few EU-fish production value chains, sufficient monitoring data are not available. Therefore it is not possible to identify which fish species and fishing grounds present a health hazard with respect to the presence of allergenic parasites. Indeed, apart from recent findings in *P. marinus* (Bao et al. 2013), no data are available to confirm that no viable parasites or their allergens are present in fishery products derived from anadromous fish species caught in freshwater ecosystems of the NW Iberian Peninsula.

The role of shad species as transport hosts for parasites from the marine to the freshwater ecosystem is also very noticeable and the transport in the opposite direction may also occur. In this regard, Bao et al. (2013) suggested the possibility that post-metamorphic juvenile of *P. marinus* might act as paratenic host of *Anisakis* spp., transporting them from freshwater to seawater in NW Iberian Peninsula waters. In addition, the haematophagus feeding of post-metamorphic *P. marinus* on both *A. fallax* (Silva et al. 2013) and *A. alosa* (Silva et al. 2014) has been documented in this region. Furthermore, it was demonstrated that European otter, *Lutra lutra* (Linnaeus, 1758), predate on shad species (Aprahamian et al. 2003) and they were also reported as an accidental host of *Anisakis* spp. in one Spanish River (Torres et al. 2004), so wildlife risks for terrestrial mammals should be considered. Likewise, Shields et al. (2002) reported infection by *A. simplex* in the American shad, *Alosa sapidissima* (Wilson, 1811), in two Oregon Rivers. On this occasion, the authors suggested that this parasite-host relationship has led to an ecological expansion of *Anisakis* spp. into rivers and may present an emerging health risk for wildlife and human consumers.

Overall, the results stress that anadromous fish species may be a significant source of gastroallergins and represent an ecological transport mechanism that transfers the parasite risk from the sea to the freshwater ecosystem.

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Authors declare that the present submission has no conflict of interest and that it complies to the ethical standards of the journal.

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TABLES

Table 1. Sampling batches. Aa: *Alosa alosa*; Af: *Alosa fallax*; n: number of individuals

* Method of inspection: adult specimens were inspected for *Anisakis* nematodes following the visual scheme established by the European Regulation EU 853.

Table 2. Percentages (%) of *Anisakis* larvae (n=72) molecularly identified by genetic markers as *A. alosa* and *A. fallax* caught in different Rivers.

Table 3. Quantitative descriptors of *Anisakis* spp. larvae. P: Prevalence; mA ± SD: mean abundance and standard deviation; mI ± SD: intensity and standard deviation; n: Number of fish sampled; TL ± SD, cm: total length and standard deviation; TW ± SD, g: total weight and standard deviation. Abundance range and intensity range are presented between brackets.

Table 1

Species	Batch	n	River	Date	Fishing method	Fish organs inspected	Method of inspection
Aa	1	160	Minho	March to August, 2009 to 2011	Trammel net	Visceral cavity and stomach	Visual
Aa	2	9	Minho	6th May 2013	Trammel net	Gill, visceral cavity, stomach and flesh	Visual and artificial digestion (visceral cavity, stomach and flesh)
Aa	3	9	Mondego	14th May 2012 (5 fish) and 15th May 2013 (4 fish)	Trammel net	Visceral cavity and stomach	Visual
Af	4	148	Ulla	March to July, 2011 and 2012.	Trammel net	Visceral cavity and flesh (only 18 fish)	Visual and artificial digestion (of flesh)
Af	5	6	Ulla	1st May to 8th June 2008	Sport fishing	Visceral cavity, stomach and flesh	Visual and artificial digestion
Af	6	27	Minho	29th April 2011	Trammel net	Visceral cavity, stomach and flesh (only 7 fish)	Visual and artificial digestion (of flesh)
Af	7	42	Minho	14th May 2012	Trammel net	Gills, visceral cavity, stomach and flesh	Visual and artificial digestion

Table 2

Alosa alosa			
Sampling River	n *Anisakis* larvae	*A. pegreffii*	*A. simplex* s.s.
Minho	33	57.58%	42.42%
Mondego	13	69.23%	30.77%
Alosa fallax			
Ulla	14	64.29%	35.71%
Minho	12	66.67%	33.33%

Table 3

Alosa alosa

Batch 1 (n= 160). Visual methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	51	63.32 ± 4.34	2428 ± 656.02	76.57%	97.88 ± 135.85 [0-509]	128.00 ± 142.58 [1-509]
Viscera	51	63.48 ± 4.68	2378 ± 743.92	96.08%	180.73 ± 161.53 [0-796]	188.10 ± 160.51 [10-796]
Whole viscera	144	64.40 ± 3.89	2351 ± 721.15	95.83%	143.81 ± 166.92 [0-877]	150.06 ± 167.74 [1-877]

Batch 2 (n=9). Visual plus enzymatic digestive methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	9	63.93 ± 5.92	2711.11 ± 727.92	66.66%	5.56 ± 9.25 [0-29]	8.33 ± 10.44 [1-29]
Viscera				100%	611.67 ± 344.03 [108-1137]	611.67 ± 344.03 [108-1137]
Whole viscera				100%	617.22 ± 348.24 [108-1137]	617.22 ± 348.24 [108-1137]
Flesh				88.88%	3.56 ± 4.10 [0-13]	4.00 ± 4.14 [1-13]
Total				100%	620.78 ± 348.82 [108-1138]	620.78 ± 348.82 [108-1138]

Batch 3 (n=9). Visual methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	9	55.33 ± 2.87	1731.11 ± 221.06	22.22%	0.22 ± 0.44 [0-1]	1 ± 0 [1]
Viscera				100%	215.00 ± 209.19 [4-588]	215.00 ± 209.19 [4-588]
Whole viscera				100%	215.22 ± 209.23 [4-588]	215.22 ± 209.23 [4-588]

Alosa fallax

Batch 4 (n=148). Visual methodologies. In addition, the flesh of 18 fish was subjected to enzymatic digestion.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Viscera	148	45.20 ± 5.24	830.59 ± 330.37	3.38%	1.61 ± 9.89 [0-89]	47.80 ± 28.86 [14-89]
Flesh	18	42.06 ± 4.12	508.14 ± 147.74	0%	0	0

Batch 5 (n=6). Visual plus enzymatic digestive methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	6	39.55 ± 5.39	643.43 ± 275.10	0%	0	0
Viscera = Total				83.33%	44.17 ± 51.17 [0-121]	53.00± 51.85 [1-121]
Flesh				16.66%	0.17 ± 0.41 [0-1]	1 ± 0

Batch 6 (n=27). Visual methodologies. In addition, the viscera and flesh of 7 fish were subjected to visual and enzymatic digestive methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	26	38.15 ± 3.43	497.12 ± 181.74	0%	0	0
Viscera	27	38.16 ± 3.37	497.04 ± 178.21	22.22%	1.26 ± 3.36 [0-13]	5.67 ± 5.35 [1-13]
Whole viscera*	7	37.29 ± 1.67	441.43 ± 48.21	14.29%	0.57 ± 1.51 [0-4]	4 ± 0
Flesh				0%	0	0

Batch 7 (n=42). Visual plus enzymatic digestive methodologies.

Organ	n	TL ± SD	TW ± SD	P	mA ± SD [range]	mI ± SD [range]
Stomach	40	36.73 ± 2.65	494.40 ± 93.36	10%	0.15 ± 0.53 [0-3]	1.50 ± 1.00 [1—3]
Viscera				60%	5.55 ± 10.60 [0-47]	9.25 ± 12.44 [1-47]
Whole viscera = Total	42	36.70 ± 2.58	493.45 ± 91.21	64.29%	5.52 ± 10.39 [0-47]	8.59± 11.96 [1-47]
Flesh				0%	0	0

* Zero larvae in stomach.

FIGURE LEGENDS

Figure 1. Map of the study area showing the location of the three rivers.

Figure 2. Accumulation of anisakid larvae at the posterior end of the terminal blind sac of an *A. alosa* stomach from the River Minho.

Figure 3. Several *Anisakis* spp. larvae accumulated outside the posterior end of the terminal blind sac of the stomach of *A. fallax* fished in the River Minho.

Figure 4. Left to right are relationships between number of *Anisakis* spp. larvae found in the visceral cavity (including stomach) of shads, using visual methods, and explanatory variables as visualized by fitting GAMs. Smoothers for the effect of total length (cm), fraction of the calendar year (yearfrac) and residuals of condition factor (res k) of *A. alosa* (Fig. 5A) and *A. fallax* (Fig. 5B).

FIGURE

Fig.1

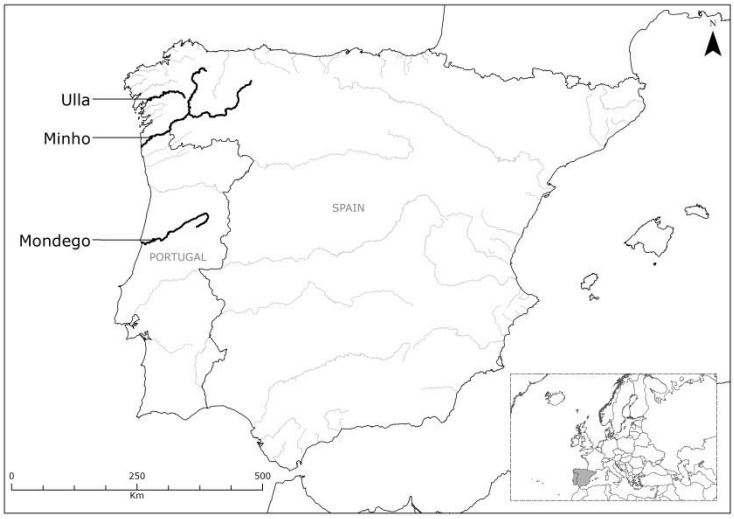


Fig. 2

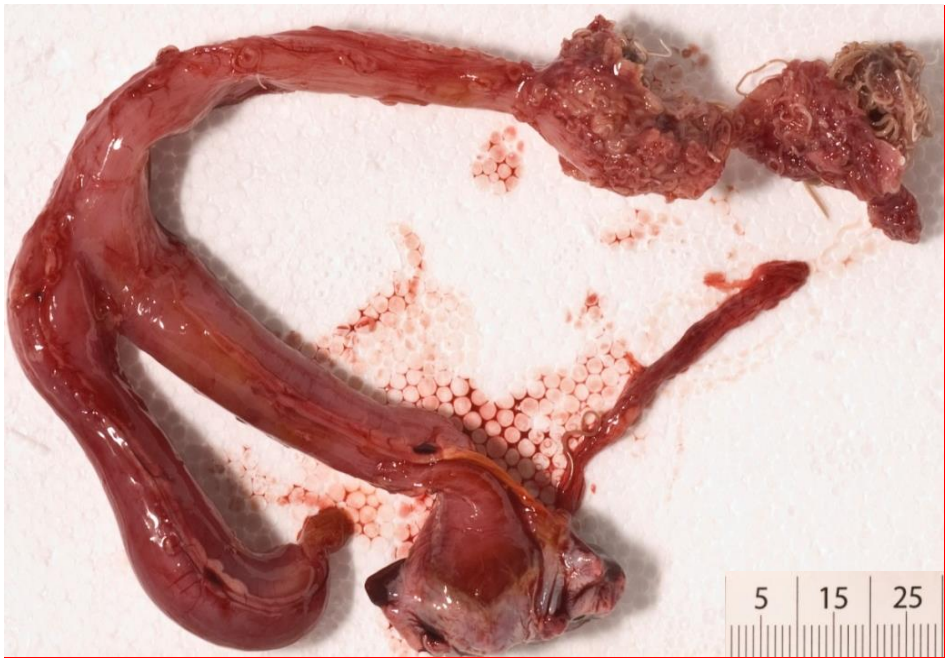


Fig. 3

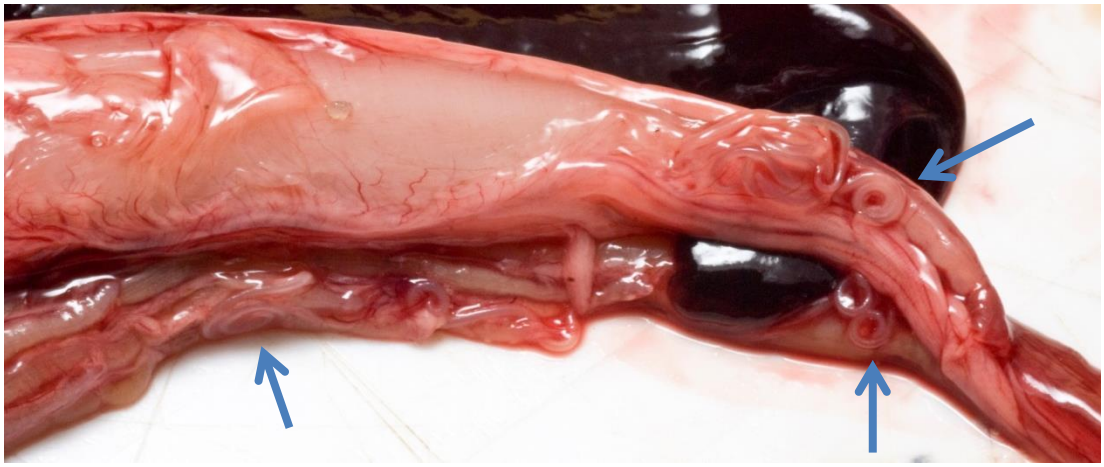
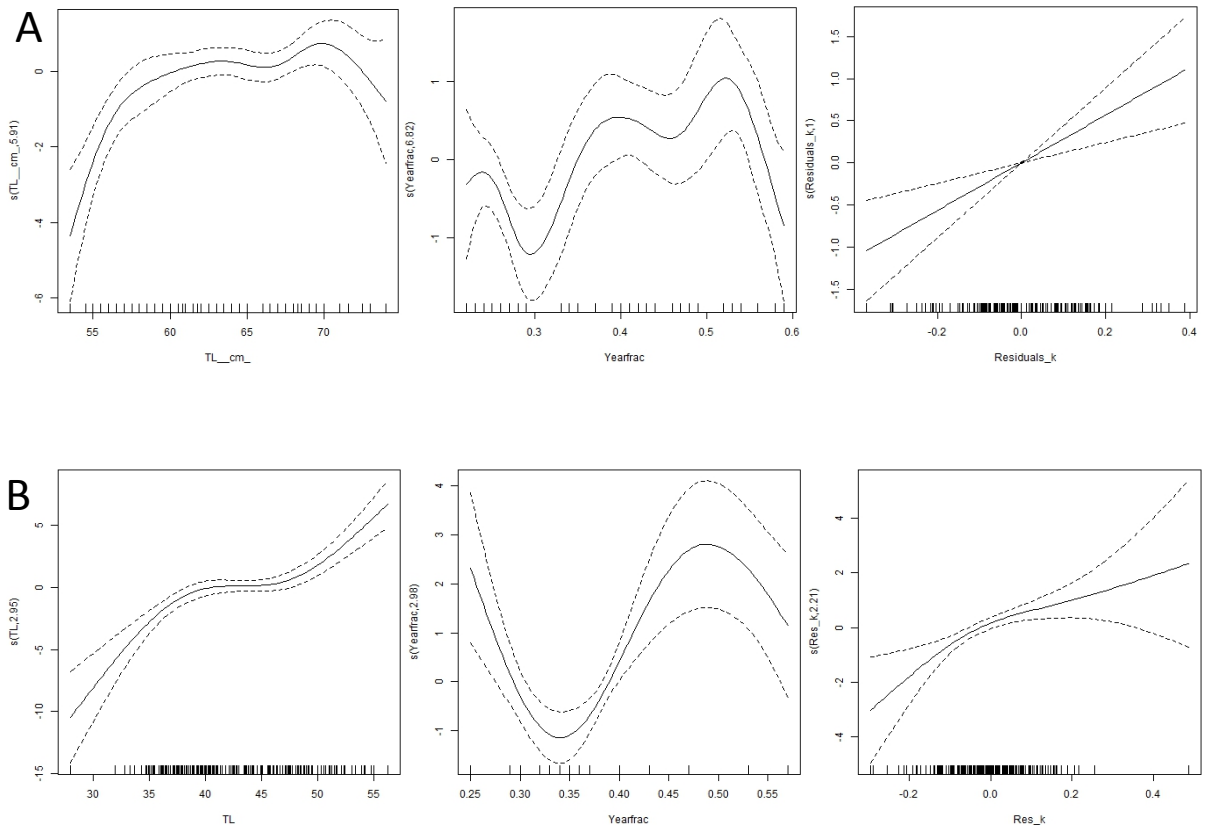


Fig. 4


A
s(TL__cm_,5.91)
TL__cm_
s(Yearfrac,6.82)
Yearfrac
s(Residuals_k,1)
Residuals_k
B
s(TL,2.95)
TL
s(Yearfrac,2.98)
Yearfrac
s(Res_k,2.21)
Res_k