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A survey of zoonotic nematodes of commercial key fish species from major European fishing grounds—Introducing the FP7 PARASITE exposure assessment study

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ABSTRACT

Harvesting and exploiting limited fisheries resources in a sustainable manner also implies achieving maximum added value from the raw material. However, the presence of parasites in the products may adversely affect consumer perception and/or pose a direct health hazard. As a major stepping-stone of the PARASITE project, an epidemiological survey was carried out to provide the basis for analysis and prediction of consumer exposure risk due to the presence of anisakid nematodes in fish from European wild-catch fisheries. The project consisted of nine separate workpackages (WP) where the exposure risk assessment survey was organized within WP2. The surveillance task also provided the data or samples needed for data management and sample storage (WP3, Biobank), molecular and genetic parasite species identification (WP4), and statistical modelling and inference (WP8). In total 17,760 fish belonging to 16 teleost species were examined for anisakids, with special emphasis on economically and ecologically important species such as Atlantic mackerel, herring, European hake, Atlantic cod and anchovy. The target fish species were sampled at four major European fishing areas including the Barents Sea, North Sea, Baltic Sea, Grand Sole Bank, waters off NW Spain and Portugal, central and western parts of the Mediterranean Sea, and the Adriatic Sea. Thus, the survey represents the largest and most comprehensive epidemiological data compilation of anisakids ever generated in terms of geographical range as well as number of fish host species and sample size. An important requirement of the survey was the use of commonly accepted nematode detection methods, i.e. the UV-press method or artificial digestion, to quantify infection level and spatial distribution of anisakid larvae in the target fish species. The basic layout, set-up and principles of the method, along with a discussion of possible source of errors are described. Additionally, the molecular and genetic markers which were used to identify and characterize different species and populations of anisakids from the targeted fish host species and geographical areas, are reviewed as well. Some basic parasite infection characteristics of each fish host species, and any relationships with the presumably most important infection predictors, i.e. fish host body size and fishing locality, are presented and discussed. Emphasis is put on anisakid occurrence in the flesh of the fish. Based on the findings, a graphical exposure risk profile is introduced, including fish species or products thereof, which due to common processing or preparation practices, are at highest risk to act as source of anisakiasis in Europe.

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1. Introduction

The EU fisheries industry is the fifth largest in the world. In the European Union, close to 5 million tons of wild fish catches are processed every year. Fishing and fish processing provide jobs for at least 275,000 people. Moreover, the EU is among the leading fish markets in the world with imports accounting for approximately €21 billion in 2014, more than 40% of world fish imports in value, with increasing trend. It should be noted, however, that Norway as an EU third country accounts for 23% of the EU seafood imports alone. The average consumption of fisheries products in the EU-28 countries was 24.9 kg/person in 2011. The annual per capita consumption rate varies greatly, however, from 5.3 kg in Hungary to 56.8 kg in Portugal (European Commission, 2016).

Nevertheless, consumers expect safe and healthy fish and fishery products. However, some of the most important fish species caught by the European fishing industries are at risk of carrying parasites when put on the market. In Europe, anisakid nematodes are the most relevant group of parasites in terms of consumer health risk and product quality, with *Anisakis* and *Pseudoterranova* as the genera of greatest concern because several species are considered a human health hazard (Mattiucci et al., 2017a). The term anisakiasis refers to the zoonotic disease provoked through accidental ingestion of viable larvae of certain *Anisakis* species which infect the edible parts of fish or squid. Among the nine nominal species belonging to the genus *Anisakis* (Mattiucci and Nascetti, 2008; Mattiucci et al., 2014), *A. simplex (sensu stricto)* and *A. pegreffii* have been confirmed to cause disease in humans (D'Amelio et al., 1999; Umehara et al., 2007; Mattiucci et al., 2011; , 2013; Lim et al., 2015; Mladineo et al., 2016; Bao et al., 2017). It was further demonstrated that *A. pegreffii* may provoke gastric (GA), intestinal (IA) and gastro-allergic anisakiasis (GAA) (Mattiucci et al., 2011, 2013; Lim et al., 2015; Mladineo et al., 2016), while both *A. simplex* (*s. s.*) and *A. pegreffii* larvae may cause allergic reactions in humans (Daschner et al., 2000). Although international regulations, e.g. EU No. 1276/2011, demand deep-freezing for at least 24 h of any fishery product to be consumed raw or semi-raw, this so-called freezing requirement is not necessarily practiced by private households or local guesthouses and restaurants. Thus, consumption of local or privately prepared dishes based on fresh, only lightly processed fish such as boquerones in Spain and marinated anchovies in Spain and Italy, probably represents a major source of anisakiasis in Europe.

Self-control programs such as HACCP (hazard analysis and critical control points) procedures in the fish industry are hampered by the fact that the epidemiology of anisakid parasites in fish caught and marketed in Europe is not well understood. The collection of data on the complete life cycle, geographical and seasonal distribution, prevalence, intensity, and infection site of parasites of public health importance in wild fish stocks and fishery products has so far been based mainly on non-systematic and opportunistic sampling, lacking appropriate monitoring programs coordinated on a pan-European scale. Therefore, a systematic epidemiological survey of the economically most important fish species and stocks from European fishing grounds could provide the basis for analyzing and modelling parasite prevalence and abundance.

In fish, the majority of *Anisakis* larvae are typically seen as whitish to greyish, flat and tight coils, measuring a few mm across. Larvae that reside in the fish flesh are very hard to detect by the naked eye since they are often transparent and may have penetrated deeply into the fillets. Moreover, the larval occurrence in terms of their abundance and spatial distribution seems largely to depend on fish host species and their respective feeding behavior. Thus, piscivorous species such as adult hake and cod are usually more heavily infected with anisakid larvae compared to strict plankton feeders such as sardine, anchovy and capelin (for reviews of the literature, see Mladineo and Poljak, 2014; Šimat et al., 2015; Cipriani et al., 2016; Levsen et al., 2016; Zorica et al., 2016). However, we know only little about the spatial distribution of anisakid larvae in various economically important fish species, i.e. where in the fish the larvae primarily reside. This is especially important whenever anisakid larvae occur in the flesh (fillets and belly flaps) of fish.

The main objective of the anisakid exposure assessment workpackage (WP2) of the PARASITE project was to provide comprehensive and comparable epidemiological data with respect to zoonotic parasites in the economically most important fish species or stocks originating from major European fishing grounds. The study focused on *Anisakis* species (mainly *A. simplex* and *A. pegreffii*), extending to other species such as *Pseudoterranova decipiens* (*s. l.*), *Contraceacum osculatum* (*s. l.*) and *Hysterothylacium aduncum* (non-zoonotic species but may have aesthetical quality reducing effect if present abundantly), where adequate data were available. Thus, the current report provides a basic overview of the methods which were commonly applied to detect anisakid nematodes in the actual fish samples, and to identify them molecularly to species level. The report further summarizes some basic epidemiological results with emphasis on larval occurrence in the fish flesh, and analyses through GAM modelling the relationships between larval occurrence and fish host body size which is already known to act as important driver of anisakid infection patterns in many fish species and fishing areas. Finally, we introduce a graphical exposure risk profile based on prevalence data of *Anisakis* spp. in the flesh of several fish species which are commonly prepared and consumed in a raw or only lightly processed state.

2. Material and methods

2.1. Target fish species

The primary decision criteria for the target fish species of the survey concerned their importance in terms of: 1) annual consumption volume/sales value, 2) significance for the fresh fish market, 3) basis for raw or semi-raw products such as sushi and sashimi, and 4) parasite history (e.g., former RASFF –Rapid Alert System for Food and Feed – notifications). Thus, the pelagic fish species included in the survey were herring (*Clupea harengus*), sardine (*Sardinus pilchardus*), anchovy (*Engraulis encrasicolus*), Atlantic mackerel (*Scomber scombrus*), chub mackerel (*S. colias*) and blue whiting (*Micromesistius poutassou*). European hake (*Merluccius merluccius*), haddock (*Melanogrammus aeglefinus*), Atlantic cod (*Gadus morhua*) and monkfish (*Lophius piscatorius* and *L. budegassa*), in addition of two flatfish species – plaice (*Pleuronectes platessa*) and four-spotted megrim (*Lepidorhombus boscii*) – represented species preferring demersal habitats. On a smaller scale, or whenever available, we also investigated whiting (*Merlangius merlangus*), European sea bass (*Dicentrarchus labrax*) and silver scabbardfish (*Lepidopus caudatus*) since these, too, are commercially utilized on an industrial scale and are of importance in a number of major European seafood markets including Spain, UK, Italy and France. Some of the fish species to be included in the survey, e.g. Atlantic mackerel, cod and hake, occur and are commercially utilized in several of the present NE Atlantic fishing areas. Thus, the epidemiological data obtained from these species and areas have been particularly analyzed for the effect of specific habitat characteristics, geographical location and fish host migration patterns on the diversity and distribution of anisakid species (see also Levsen et al., 2017; Gay et al., 2017; Pascual et al., 2017).

2.2. Sample size and fish host biometric data

Fish host sample size varied among host species, sampling localities and sampling date/year. In general, smaller or medium sized species, e.g. anchovy, herring, mackerel or blue whiting were sampled and examined in greater quantities compared to larger species such as Atlantic cod, haddock or monkfish (Table 1). This was partly due to the fact that processing and UV-inspection of smaller fish is less labor- and time-intensive than examining larger fish. Additionally, samples of some other fish species, e.g. European sea bass, were more costly to obtain

Table 1
Fish host species and sample size, along with number of *Anisakis* spp. subsamples for genetic species analysis, by fishing ground given as ICES/FAO fishing zones and name of sampling locality.

Fish host species, sample size:	Atlantic fishing grounds including Baltic Sea				Mediterranean fishing grounds			
	N fish	ICES area	Sampling locality	N *Anisakis* gen. ident.	N fish	FAO area	Sampling locality	N *Anisakis* gen. ident.
Atlantic mackerel, N = 1801 (*Scomber scombrus*)	526	IVa,b	North Sea	472	168	37.2.1	North Adriatic Sea	92 (total)
	231	VIId	English Channel	693	30	37.1.3	Tyrrhenian Sea	
	300	Vb1	Faroe Islands waters	56	19	37.1.1	Alboran Sea	
	300	IIa	Southeastern Norwegian Sea	272	70	37.1.2	Gulf of Lion	
	157	VIIIc; IXa	Cantabric Sea; Off NW Spain & Portugal	102				
	1514			1595	**287**			92
Chub mackerel, N = 507 (*Scomber colias*)	21	IXa	Off NW Spain & Portugal	0	344	37.2.1	Adriatic Sea	175 (total)
					100	37.1.1	Balearic Sea	
					42	37.1.3	Tyrrhenian Sea	
	21			0	**486**			175
Herring, N = 2673 (*Clupea harengus*), four stocks: North Sea (n = 1010) English Channel (n = 242) Norw. spring spawning (n = 726) Baltic Sea (N = 695)	1010	IVa,b	North Sea	1395 (total)				
	242	VIId	English Channel					
	276	IIa	Norwegian Sea					
	150	IVa	North Sea					
	300	Vb1	Faroe Islands waters					
	600	BAL24	Southwestern Baltic Sea					
	95	BAL25	Central Baltic Sea					
	2673			1395				
Sardine, N = 1704 (*Sardina pilchardus*)	140	IXa	Off NW Spain & Portugal	0	908	37.2.1	East Adriatic Sea	61 (total)
					200	37.2.2	Off South Sicily	
					356	37.1.1	Off West Sardinia	
					100	37.1.3	North Tyrrhenian Sea	
	140			0	**1564**			61
Anchovy, N = 5108 (*Engraulis encrasicolus*)	956	VIIIc	Cantabric Sea	0	645	37.2.1	North Adriatic Sea	547 (total)
					528	37.2.1	Central Adriatic Sea	
					518	37.2.1	East Adriatic Sea	
					280	37.2.2	South Adriatic-Ionian Sea	
					108	37.3.1	Aegean Sea	
					200	37.2.2	Off South Sicily	
					1323	37.1.3	Tyrrhenian-Ligurian Sea	
					550	37.1.1	Sardinian-Balearic-Alboran Sea	
	956			0	**4152**			547
Atlantic cod (*Gadus morhua*), N = 755 (incl. 234 cod of which only the flesh was examined)	146 (46)	I	Southern Barents Sea	0				
	386 (188)	BAL24, 25	Baltic Sea	76				
	130	IVa	Northern North Sea	1913				
	93	IVb	Central North Sea	0				
	755 (234)			1989				
Haddock, N = 441 (*Melanogrammus aeglefinus*)	150	I	Southern Barents Sea	0				
	291	IVa,b; VIa	North Sea; Off West Scotland	0				
	441			0				
European hake, N = 1634 (*Merluccius merluccius*)	75	IVb; VIa	North Sea; Off West Scotland	388 (total)	72	37.2.1	North Adriatic Sea	877 (total)
	188	VII	Grand Sole		137	37.2.1	West Adriatic Sea	
	242	VIIIc; IXa	Cantabric Sea; Off NW Spain & Portugal		257	37.2.1	East Adriatic Sea	
					96	37.2.2	South Adriatic-Ionian Sea	
					27	37.3.1	Aegean Sea	
					64	37.2.2	Off South Sicily	
					241	37.1.3	Tyrrhenian-Ligurian Sea	
					34	37.1.1	Off West Sardinia	

(*continued on next page*)

Table 1 (*continued*)

Fish host species, sample size:	Atlantic fishing grounds including Baltic Sea				Mediterranean fishing grounds			
	N fish	ICES area	Sampling locality	N *Anisakis* gen. ident.	N fish	FAO area	Sampling locality	N *Anisakis* gen. ident.
					49	37.1.2	Gulf of Lion	
					152	37.1.1	Balearic-Alboran Sea	
	505			388	**1129**			877
Blue whiting, N = 1534 (*Micromesistius poutassou*)	453	IVa; VIa;	North Sea incl. Faroe waters	124	172	37.2.1	East Adriatic Sea	134
	454	Vb VIa,b	Rockall	0	69	37.1.1	Alboran Sea	
	133	VIIIc	Cantabric Sea	0				
	253	IXa	Off NW Spain & Portugal	37				
	1293			161	**241**			134
Whiting, N = 517 (*Merlangius merlangus*)	291	IVa; IVb	North Sea	0				
	226	VIa	Off West Scotland	0				
	517			0				
Plaice, N = 464 (*Pleuronectes platessa*)	240	VIId, IVc	English channel/S North Sea	2				
	12	IVa	Northern North Sea	9				
	102	IVb	Central North Sea	0				
	110	VIa	Off West Scotland	0				
	464			11				
Four-spotted Megrim, N = 287 (*Lepidorhombus boscii*)	48	IXa	Off NW Spain & Portugal	0				
	139	VIIj,k	Grand Sole	0				
	100	VIIIc	Cantabric Sea	0				
	287			0				
Monkfish (*Lophius* spp.), N = 211	58	VIIj,k;	Grand Sole; Cantabric	157 (total)	6	37.1.1	Alboran Sea	44 (total)
L. piscatorius, n = 64	83	VIIIc; IXa	Sea; Off NW Spain & Portugal		29	37.1.1	Alboran Sea	
L. budegassa, n = 147		VIIj,k;	Grand Sole; Cantabric Sea; Off NW Spain & Portugal		30	37.1.1	Balearic Sea	
		VIIIc; IXa			5	37.2.1	Adriatic Sea	
	141			157	**70**			44
Silver Scabbardfish, N = 86 (*Lepidopus caudatus*)					35	37.2.1	Alboran Sea	398 (total)
					29	37.1.3	Tyrrhenian Sea	
					22	37.2.2	Off South Sicily and Malta	
					86			398
European sea bass, N = 38 (*Dicentrarchus labrax*)					17	37.1.1	Balearic Sea	3 (total)
					8	37.1.3	Tyrrhenian Sea	
					13	37.2.1	Adriatic Sea	
					38			3
Total, N = 17,760	**9707**			5696	**8053**			2331

which again restricted their sample size.

For each fish, total round body weight (TW in g) and three body length measures (mm), i.e. total body length (TL), fork length (FL) and standard length (SL) were recorded. For statistical analyses of the relationships between parasite infection and host biometric parameters, TW and/or TL were usually used as a descriptor of fish size. Other host biometric parameters that were recorded on a routine basis included fish gender, state of maturity, liver- and gonad weight (when feasible), as well as gross identification of stomach contents (empty/fish/crustaceans/mollusks/mud). TW and TL were also used to derive Fulton's condition index K ($K = W*10^5/L^3$).

2.3. Sampling areas and sampling procedures

Fish (N = 17,760) were sampled from 2012 to 2015 at various European fishing grounds. Fish were obtained during research cruises or regular commercial fishing operations and either processed and examined freshly on board the vessels, or deep-frozen immediately after catch for further processing and inspection at the respective laboratories on land. Some fish samples were also obtained at local fish markets or from local fishermen. Fig. 1 shows the ICES (Atlantic) or FAO (Mediterranean) fishing zones, highlighted dark-blue, in which the present fish samples were collected, while Table 1 provides an overview of the subsamples per fish species and particular ICES or FAO zone, along with names of the respective sampling areas.

2.4. Parasite inspection methods

In order to ensure best possible comparability of the nematode infection data, an important premise of the survey was, whenever feasible, to apply the same nematode inspection method throughout (only one surveyor applied the artificial digestion method, see Llarena-Reino et al. (2013) and references therein). This strategy probably reduced the effect of errors due to differences in detection efficiency and operator skills on the quality of data during sampling and parasite recording.

The UV/press-method is increasingly applied during systematic detection of nematode larvae in the flesh of fish, especially in large-scale scientific surveys (Levsen and Lunestad, 2010; Levsen and Karl, 2014; Klapper et al., 2015; Cipriani et al., 2016; Levsen et al., 2016). The method utilizes the fluorescence of frozen anisakid larvae (Pippy, 1970) and is based on visual inspection of flattened/pressed and subsequently deep-frozen fish fillets or viscera under UV-light (Karl and

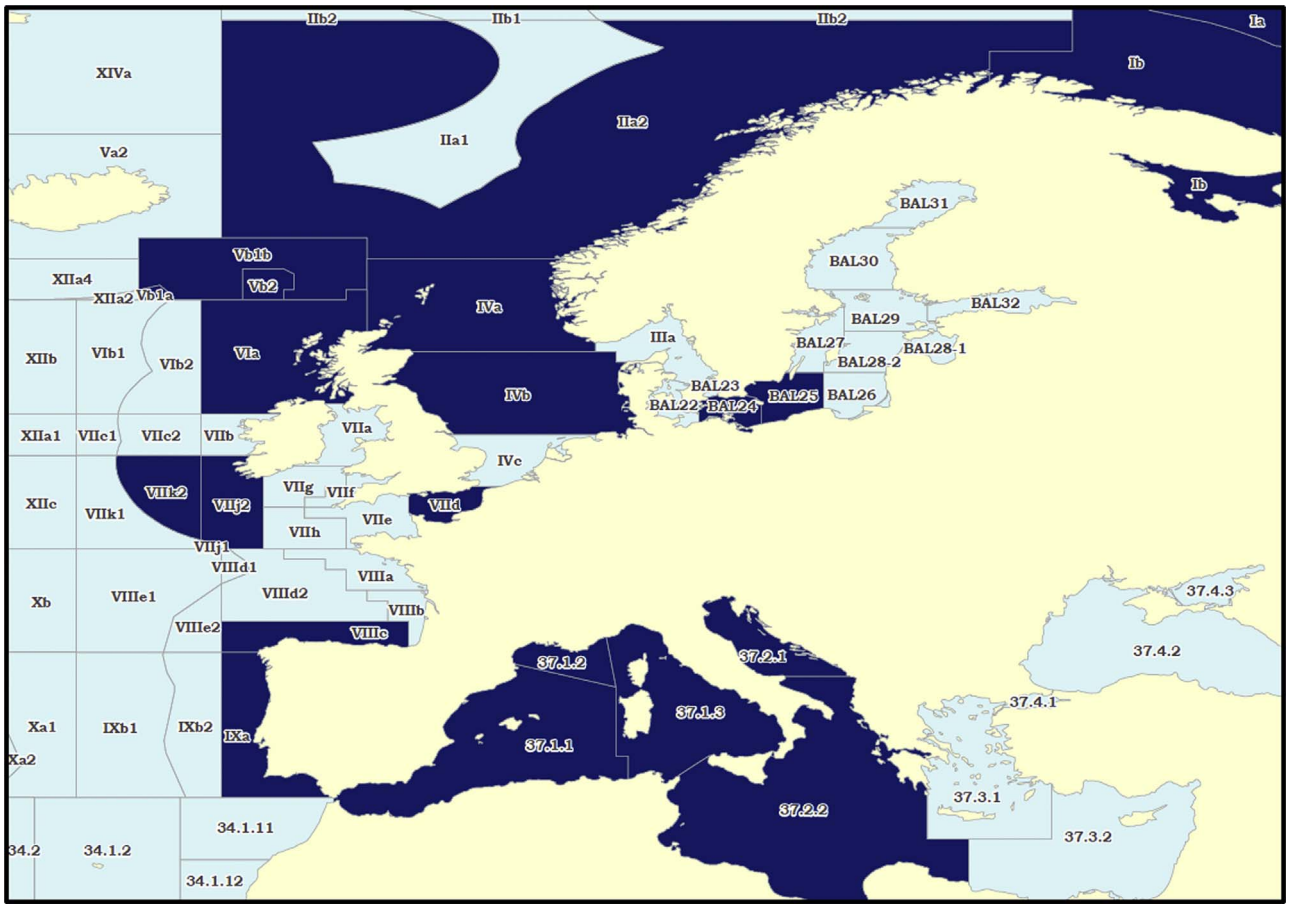

Fig. 1. ICES and FAO Northeast Atlantic fishing zones; the present fish sampling zones are highlighted dark-blue. I: Southern Barents Sea; IIa: Southeastern Norwegian Sea; IVa,b: North Sea; Vb: Faroe Islands waters; VIa,b: Rockall; VIIj,k: Grand Sole; VIId: English Channel; VIIIc: Cantabric Sea; IXa: Off NW Spain & Portugal; BAL 24: Southwestern Baltic Sea; BAL 25: Central Baltic Sea; 37.1.1: Western Mediterranean; 37.1.2: Gulf of Lion; 37.1.3: Tyrrhenian Sea; 37.2.1: North Adriatic Sea; 37.2.2: South Adriatic-Ionian Sea; 37.3.1: Aegean Sea.

Leinemann, 1993). Like many other eukaryote organisms, anisakid nematodes accumulate lipofuscin, an auto-fluorescent pigment, within their cells. When the parasite cells break, the lipofuscin is released and upon excitement of the larvae with UV light, they fluoresce much brighter than the surrounding fish flesh. Prior to the pressing process, each fish is gutted and manually filleted before placing the visceral organs and both left and right-side flesh (fillets incl. belly flaps) into clear plastic bags. The samples are then pressed to 1–2 mm thick layers in a hydraulic or pneumatic pressing device (holding time approx. 5 s at 800–1400 kPa). The bags containing the pressed fillets or viscera are then deep-frozen prior to visual inspection under a 366 nm UV-light source equipped with both up- and down-light. Any anisakid larvae present appear as fluorescent spots in the samples; the brightness probably depending on various factors such as anisakid species involved, their size and age, the extent of encapsulation, and possibly, if the freezing-thawing cycle affects the integrity of the larvae.

Another advantage compared to the other widely used nematode inspection method, i.e. artificial digestion of soft tissue in an aqueous pepsin/HCl-solution, is that the UV-press method allows determination of the approximate larval infection site in both the flesh and the viscera. Thus, to facilitate the screening of the fish flesh, each flesh side (fillet + belly flap) is divided into 4 sections in the following manner; anterior ventral (AV) which corresponds roughly to belly flap, anterior dorsal (AD), posterior ventral (VP) and posterior dorsal (DP) (Fig. 2). After pressing, the different sections of each flesh side are readily recognized since – depending on fish species – the lateral line or red muscle area, or both, may be used as reference points/axis (Fig. 3). Whenever larger fish such as cod, haddock or monkfish were examined, each fillet or fish side was cut into smaller parts which were processed separately.

The nematode detection efficiency of the UV-press method was recently evaluated and compared with the artificial digestion method (Pepsin/HCl) in a ring trial (see Gómez-Morales et al., 2017). The results showed that the number of *Anisakis* spp. larvae recovered by the UV-press method had higher level of agreement (90%) with the number of spiked larvae compared with the number of larvae recovered by artificial digestion (83%).

The epidemiological data sets were organized as separate Microsoft Excel-workbooks per fish species examined. Each data set was consecutively updated according to the results from the genetic identification whenever new data were available. The data sets were

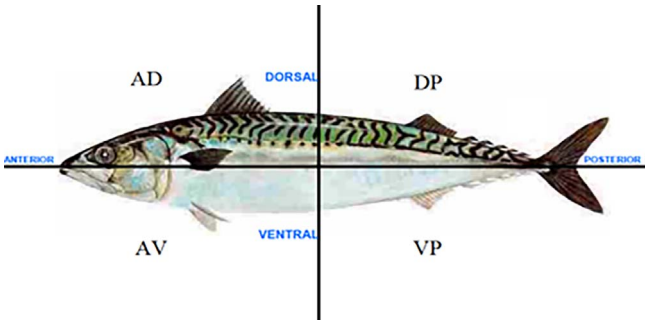

Fig. 2. Anatomical body sections of fish, exampled by a mackerel, used for parasite infection site recording when applying the UV-press nematode inspection method.

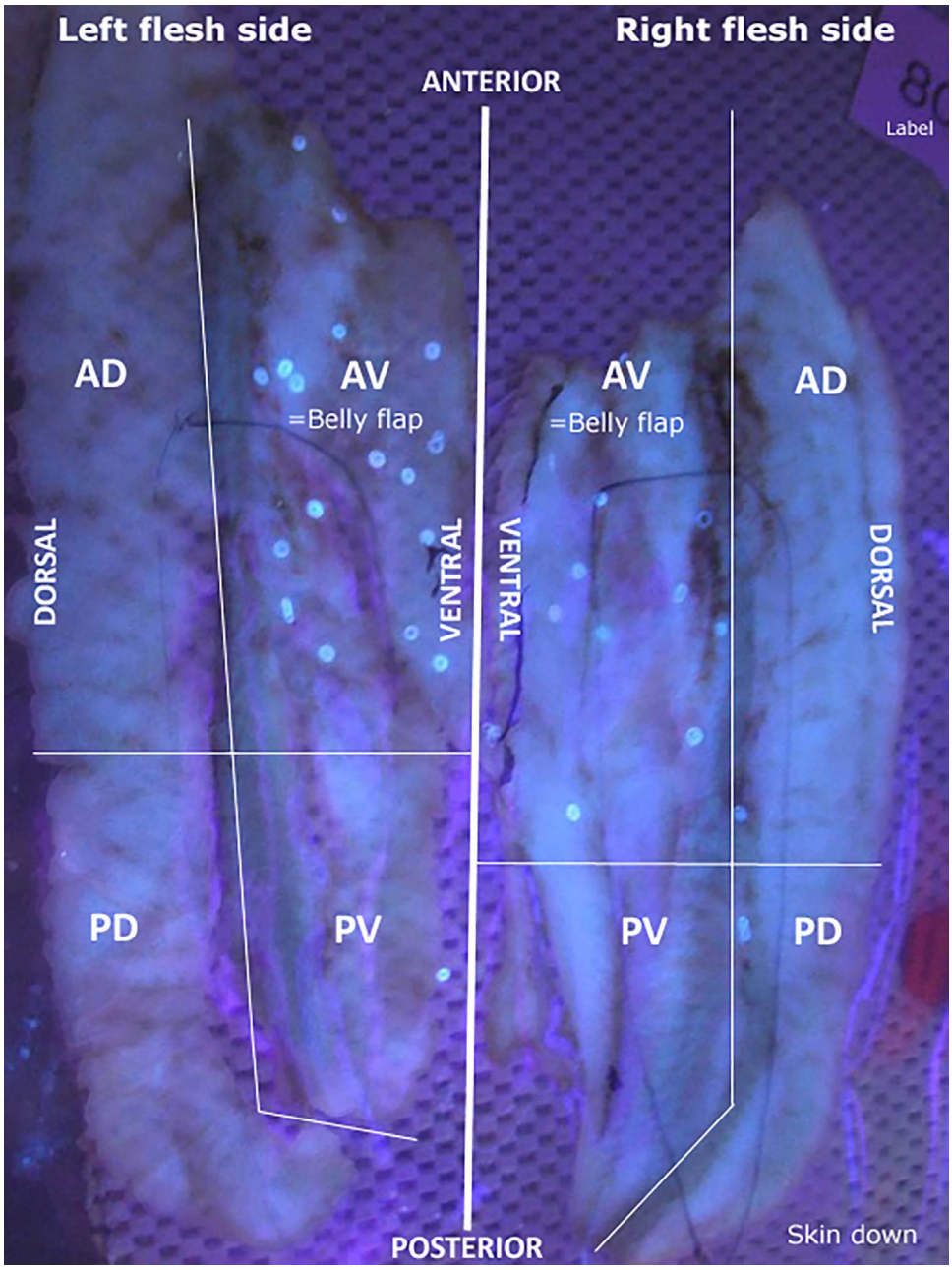

Fig. 3. The four different muscle sections of the pressed flesh sides of a blue whiting. Abbreviations: AV – anterior ventral (=belly flap), AD – anterior dorsal, PV – posterior ventral, PD – posterior dorsal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subsequently transferred into the PARASITE BioBank data platform. In this way, the epidemiological data per fish species/stock and catching area, including *Anisakis* sibling species distribution, were available for geo-referenced data management.

2.5. PARASITE biobank

All fish host and parasite data generated during the PARASITE project were included in a biobank (BB) platform which was created to ensure traceability of the samples and associated data. The biobank consists of four nodes held in Vigo – Spain (repository and central node), Rome – Italy (DNA data and samples) and Madrid – Spain (protein and sera). The service is based on a non-profit scheme and includes data of more than 14000 specimens of different fish and cephalopod species, largely surpassing the collection of 220 000 zoonotic parasites. Since 2015, the platform is ISO certified as ISO 9001 (for details, see González et al., 2017).

2.6. Genetic nematode species identification

After initial gross identification to genus level based on morphology, various subsamples of ascaridoid nematodes collected from all target fish hosts and sampling localities included in the survey, were shipped in a frozen state to the Department of Public Health and Infectious Diseases of the Sapienza-University of Rome, for subsequent genetic identification. Samples of worms were stored frozen at −50 °C prior to analysis. Individual multi-locus genotypes of the ascaridoids were obtained by using distinct types of molecular markers. For specific identification of the nematodes of the genus *Anisakis*, the following genetic/molecular markers were applied: allozymes, sequence analysis of the mitochondrial *cox2* (mtDNA *cox2*) gene, and sequence analysis of the elongation factor EF1 α-1 nuclear DNA gene. For specific identification of the nematodes of the genera *Pseudoterranova* and *Contracaecum*, sequence analysis of the mitochondrial *cox2* (mtDNA *cox2*) was used. Finally, the nematodes of the genus *Hysterothylacium* were identified by sequence analysis of the internal transcribed spacers (ITS rDNA) region.

For allozyme analysis of *Anisakis* spp. larvae, standard horizontal starch gel electrophoresis was performed to analyze the variation at four allozyme loci of diagnostic value in *Anisakis* species (Mattiucci et al., 1997, 2014). The actual loci were, 1) adenylate kinase (*Adk-2*, EC 2.7.4.3), 2) leucine-alanine peptidase (*Pep C-1, Pep C-2*, EC 3.4.11), and, 3) superoxide dismutase (*Sod-1*, EC 1.15.1.1). Genetic analysis of the allozyme data was performed using BIOSYS-2 software, while any deviation from the Hardy-Weinberg equilibrium was estimated with a $\chi 2$ test. The tissue homogenates of *Anisakis* spp. larvae from the starch gel electrophoresis, were preserved at −20 °C and subsequently used to extract genomic DNA from each individual larva examined. Total DNA was extracted using the cetyltrithylammonium bromide method (CTAB) (for details, see Mattiucci et al., 2014), or with the DNeasy® Blood & Tissue 120 kit (Qiagen) following the manufacturer's instructions. DNA was subsequently quantified by using the Qubit™ dsDNA HS Assay Kit with Qubit 2.0 (Invitrogen™).

The mitochondrial cytochrome c oxidase subunit II (*cox2*) gene was amplified using the primers 211F (5′-TTTTCTAGTTATATAGATT GRTTYAT-3′) and 210R (5′-CACCAACTCTTAAAATTA TC-3′), as previously reported by Mattiucci et al. (2014, 2015) and Timi et al. (2014) for the species of the genera *Anisakis, Contracaecum* and *Pseudoterranova*, respectively. Polymerase chain reaction (PCR) was carried out according to the procedures described by Mattiucci et al. (2014) and Timi et al. (2014). The sequences obtained for the mtDNA *cox2* gene in the present study, were analyzed and aligned with the sequences of the same gene from other previously characterised anisakid species using GenBank Blast software and ClustalX (Thompson et al., 1997).

For the elongation factor (*EF1 α-1* nDNA) nuclear gene which was studied in the sibling species of the *A. simplex (s. l.)* complex, the primers EF-F (5′-TCCTCAAGCGTTGTTATCTGTT-3′) and EF-R (5′-AGTTTTGCCACTAGCGGTTCC-3′) were used (see Mattiucci et al., 2016). PCRs were carried out in a 25 µl volume containing 0.5 µl of each primer 10 mM, 2.5 µl of $MgCl_2$ 25 mM (Promega), 1.5 µl of 5 x buffer (Promega), DMSO 0.08 mM, 0.5 µl of dNTPs 10 mM (Promega), 5 U of Go-*Taq* Polymerase (Promega) and 2 µl of total DNA. PCR temperature conditions were as follows: 94 °C for 3 min (initial denaturation), followed by 35 cycles at 94 °C for 45 s (denaturation), 58 °C for 40 s (annealing), 72 °C for 1 min (extension) and followed by post-amplification at 72 °C for 10 min. An initial sample of 50 individuals, belonging to the two species, *A. pegreffii* and *A. simplex* (*s. s.*), previously identified by allozymes, were sequenced at the elongation factor 1 alpha 1 gene. The obtained sequences were aligned in order to detect any fixed diagnostic nucleotide positions, which would allow to separate the two species under examination (see Mattiucci et al., 2016).

Subsamples of *Hysterothylacium* spp. larvae or adults were identified to species level by sequence analysis of the internal transcribed spacers (ITS rDNA) region. PCR amplification was performed using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′), as reported by Zhu et al. (2000). PCR amplification conditions were as follows: 94 °C for 5 min (initial denaturation), followed by 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final elongation step at 72 °C for 5 min (Zhu et al., 2000). Obtained sequences were analyzed with GenBank Blast software and aligned with previously characterised sequences by applying ClustalX (Thompson et al., 1997). Phylogenetic analysis of the sequences obtained from specimens of the genera *Anisakis, Pseudoterranova, Contracaecum* and *Hysterothylacium*, was inferred with the Bayesian inference method and performed by using MrBayes (Ronquist et al., 2012) while Bayesian analysis was performed with Jmodeltest (Posada, 2008), using the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). Posterior probabilities were estimated and used to assess support for each branch in inferred phylogeny with probabilities where $P \geq 95\%$ was indicative of significant support (Reeder, 2003).

2.7. Infection data analyses

In order to account for the effect of fish size on estimates of *Anisakis* spp. prevalence and abundance, we fitted generalised additive models (GAM) to prevalence and abundance, for each species and each sampling area of the Atlantic and Mediterranean Sea. To avoid overfitting, the complexity of smoothers was restricted by setting a maximum value for bases dimension ($k = 4$). For abundance data, negative binomial or Poisson models were fitted, the latter being appropriate only for area-species combinations with low maximum abundance (< 10); for higher maximum abundance levels, Poisson model diagnostics indicated overdispersion of the abundance data. For presence-absence data, binomial models were fitted. In all cases, the default link functions were applied, i.e. log for Poisson and negative binomial models, and logit for binomial models. No models were fitted if sample size was < 20, if prevalence was < 0.05. Additionally, if prevalence was 100%, no model was fitted. Regardless of whether the size effect was significant, models were used to predict *Anisakis* spp. presence or abundance (mean and SE) in fish of (a), mean size sampled for the species and (b), mean size sampled for the modelled species-area combination. All models were fitted in R. Note that standard error values are reported to indicate confidence in the mean value, but in the case of non-normal distributions, confidence intervals must be calculated prior to back-transformation of results onto the response scale.

3. Results and discussion

The results focus mainly on occurrence and distribution of zoonotic anisakid species in the edible parts of fish, which for most species implies the flesh. However, some fish species such as sardine and anchovy, are commonly consumed round, i.e. uneviscerated, due to their small size, and often only lightly processed as in salting or marinating. This practice is common in Mediterranean countries and, although restaurants are obliged to freeze the fish prior to processing, many households prepare the dishes without prior thermal treatment (Cipriani et al., 2016). Therefore, in certain coastal regions in southern Europe, *Anisakis* spp. infections in the viscera of some fish species may represent a consumer health risk. However, for general assessment of the consumer exposure risk related to the presence of anisakid larvae in particularly relevant fish species or products thereof (see Section 3.2.4. – *Anisakis* spp. exposure risk profile), three aspects were primarily considered here; 1) larval occurrence in the fish flesh and, whenever appropriate, their preferred infection site, 2) fish host body size/length, and, 3) differences in anisakid species composition related to geographic area or fish stock. For fish or products processed mainly for the fresh fish markets, the preferred site of the larvae was recorded to assess the spatial distribution pattern as basis for advising the industry as to possible trimming of fillets in order to reduce the probability of parasite presence.

3.1. Basic Anisakis spp. infection characteristics by fish host species

3.1.1. Atlantic mackerel (Scomber scombrus)

Mackerel from off NW Spain and Portugal showed significantly higher prevalence, abundance and intensity of *Anisakis* sp. larvae compared to their North- and Norwegian Sea and Mediterranean congeners, both when considering overall infection (viscera and flesh) and infection in the flesh. The by far lowest infection levels were recorded in mackerel from the Mediterranean fishing grounds, with only around 4% larval prevalence in the fish flesh and a maximum intensity of one (1) larva. Regardless of fish host size or catching area, the largest proportions of muscle residing larvae occurred in the ventral sections of the fish flesh which for most fish species including Atlantic mackerel, comprises the belly flaps (see Levsen et al., 2017).

GAM-analyses revealed a highly significantly positive relationship between both prevalence and abundance of flesh residing *Anisakis* spp. larvae, and body size/length of mackerel from the North Sea (Fig. 4a, d). However, the relationship tended to weaken with lower latitudes, e.g. the same variables were only very weakly related in mackerel

caught off NW Spain and Portugal (Fig. 4b, e). Moreover, no significant effect of host body length on larval prevalence and abundance in the flesh was apparently present in fish caught in the southern Norwegian Sea (Fig. 4c, f) which represented the northernmost catching locality of Atlantic mackerel. Due to generally very low larval infection levels in the fish flesh, GAMs were not fitted for *S. scombrus* from any of the Mediterranean fishing grounds.

Genetic anisakid species identification revealed that *A. simplex sensu stricto* (*s. s.*) is the dominating species in mackerel from the Atlantic fishing areas. However, we recorded four *A. pegreffii* larvae in the viscera of three mackerel caught in the northernmost fishing areas, i.e. the North Sea and southern Norwegian Sea. Similarly, 11 *A. pegreffii* were identified in mackerel caught in the southern North Sea including the English Channel. In the waters off NW Spain and Portugal, *A. simplex* (*s. s.*) constitutes still the largest sibling fraction (86%), with *A. pegreffii* and *A. simplex* (*s. s.*)/*A. pegreffii* F1 hybrids occurring at much lower frequencies (11% and 3%, respectively). In the mackerel caught in the Mediterranean, *A. pegreffii* appears to be the dominating species with only three *A. physeteris* larvae detected in three individual mackerel. The findings imply that *Anisakis* sibling species may be useful supplementary biological markers to further elucidate changing migration patterns or intermixing between different spawning stocks of Atlantic mackerel (see Levsen et al., 2017).

3.1.2. Chub mackerel (Scomber colias)

Overall prevalence of anisakid larvae in chub mackerel collected in the Atlantic Ocean and Mediterranean Sea was 60.7% while overall mean intensity reached 15.3. Although the infection differed greatly between fillets and viscera, both in terms of prevalence ($p < 0.001$) and mean intensity ($p < 0.01$), 18.1% prevalence and mean intensity of 2.1 in the flesh/fillets is important from a consumer's point of view. The anterio-ventral (AV) parts of the fillets were the preferred infection site in the flesh of chub mackerel, carrying between 67% (Tyrrhenian Sea) and 100% (Atlantic waters, ICES IXa) of all muscle residing *Anisakis* spp. larvae, respectively (Table 2).

Highest overall prevalence (100%) and mean abundance (145.9 ± 84.5) was recorded in chub mackerel originating from the Central and southern Adriatic Sea. These findings illustrate the importance of fish host size as predictor of parasite occurrence in the Adriatic Sea since the chub mackerel were on average more than 12 cm longer than the other Adriatic samples. This trend was also apparent in the flesh of these fish where both prevalence (78.9%) and mean abundance (3.8 ± 2.9) were significantly higher compared to the other chub mackerel from the Adriatic Sea (n = 325; prevalence 21.2%; abundance 0.33 ± 0.81). However, a highly significantly positive effect of fish host body size/length on larval prevalence and abundance in the fish flesh was still present in pooled samples of chub mackerel from all Adriatic localities (n = 344) (Fig. 5).

3.1.3. Herring (Clupea harengus) (four stocks)

The herring samples of the Norwegian spring spawning stock (NSS), which were obtained in the southern Norwegian Sea and around the Faroe Islands, showed significantly higher prevalence and abundance of *A. simplex* (*s. s.*) larvae compared to herring of the other stocks (Table 3). The differences were most pronounced for larvae residing in the fish flesh. Both prevalence and mean abundance of muscle residing larvae in herring of the NSS stock were more than twice as high, reaching 37.1% and 0.6, respectively, compared to herring belonging to the North Sea- or western Baltic stock. However, the herring of the NSS stock were significantly larger ($p < 0.001$) than their congeners of the other stocks. Indeed, there seems to be a marked accumulation effect of fish size on *A. simplex* (*s. s.*) abundance in NE Atlantic herring. Thus, in all stocks considered here, there was a markedly positive relationship between fish host size (TL) and overall larval prevalence and abundance. The effect of fish host body length as major predictor of larval occurrence in the flesh of herring is illustrated in Fig. 6, exemplified by

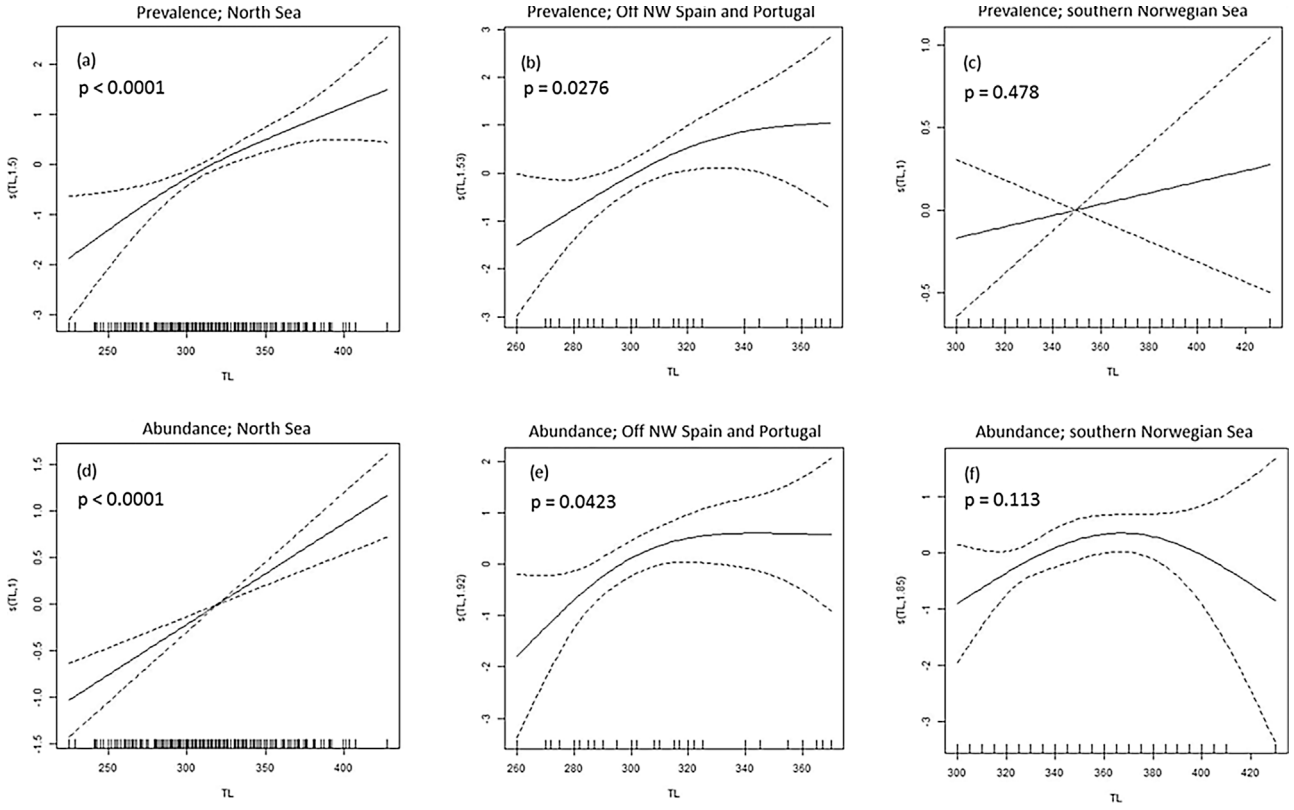

Fig. 4. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence and abundance of *Anisakis* spp. larvae in the flesh of Atlantic mackerel (*Scomber scombrus*) from the North Sea (a, d), off NW Spain and Portugal (b, e) and southern Norwegian Sea (c, f), respectively. Dashed lines represent 95% conf. intervals around the main effects.

Table 2
Sample size and basic fish host biometric data, along with basic *Anisakis* spp. infection parameters of chub mackerel (*Scomber colias*) from the Mediterranean Sea and Atlantic Ocean.

Fishing area	N fish	TL	TW	Musculature				Viscera	
				P (%)	Abund./Intensity	Rel. distr. Vtrl: Drsl	Rel. distr. Left: Right	P (%)	Abund./Intensity
Adriatic Sea (FAO 37.2.1 and 37.2.2)	344	220 ± 46 (105–400)	81 ± 35 (8–397)	24.4	A: 0.5 ± 1.3 (0–9) I: 2.2 ± 1.9 (1–9)	85: 15	53: 47	73.8	A: 12.4 ± 39.2 (0–326) I: 16.8 ± 44.9 (1–326)
Tyrrhenian Sea (FAO 37.1.3)	42	333 ± 23 (280–390)	352 ± 78 (200–589)	7.1	A: 0.1 ± 0.3 (0–1) I: 1.0 (1)	67: 33	100: 0	54.8	A: 2.7 ± 2.6 (0–10) I: 3.7 ± 2.3 (1–10)
Balearic Sea (FAO 37.1.1)	100	285 ± 20 (250–350)	206 ± 45 (130–357)	0	/	/	/	9.0	A: 0.1 ± 0.5 (0–3) I: 1.6 ± 0.7 (1–3)
Atlantic Ocean (ICES IXa)	21	286 ± 27 (250–335)	207 ± 55 (141–309)	23.8	A: 0.4 ± 0.8 (0–2) I: 1.8 ± 0.5 (1–2)	100: 0	56: 44	61.9	A: 5.8 ± 8.0 (0–26) I: 9.4 ± 8.3 (1–26)

Abbreviations;: TL − Total body length (mm); TW − Total body weight (g); P − prevalence, A − Abundance, I − Intensity, both given as mean ± SD (range); Rel. distr. − Relative distribution (%); Vtrl − ventral portion of fish flesh (corresponds roughly to belly flap); Drsl − dorsal portion of fish flesh.

the present Norwegian spring spawning- and Baltic stock samples. The findings underline the importance of proper freezing, especially of large NE Atlantic herring, before consumption in a semi-raw state such as in pickled or salted herring. However, herring caught east of Bornholm Island (ICES division BAL25) turned out to be uninfected and were hence not included in the analysis. This finding indicates that the actual fish belong to a separate Baltic herring stock entity with another migration pattern which probably excludes southern or western areas of the Baltic Sea (e.g., BAL 24) where *Anisakis* spp. fish infections commonly occur. Additionally, there were no marked differences between the herring stocks with respect to relative larval distribution in the fish flesh, i.e., between 87% and 91% of the larvae occurred in the belly flaps, with almost equal proportions lodging in the left and right flesh side (Table 3).

Genetic anisakid species identification using diagnostic allozymes and sequence analysis of the mtDNA-*cox2* gene, revealed that the entire subsample of *Anisakis* (n = 1395) collected from all herring stocks considered here, belonged to *A. simplex* (*s. s.*). However, 481 *Anisakis* spp. larvae, collected from herring sampled in the Norwegian-, Baltic- and North Sea including the English Channel, were further analyzed with respect to intraspecific genetic differentiation within the populations from the four sampling areas by applying mtDNA *cox2* sequence analysis. Genetic differentiation at population level between the different fishing areas, was estimated and compared based on molecular variance analysis and F_{st} values. Haplotype network construction showed significant differences in frequencies between samples of *A. simplex* (*s. s.*) from the actual areas. The results indicate a genetic sub-structuring of *A. simplex* (*s. s.*) from herring fished in different areas of the NE Atlantic, which again seems largely to correspond to the different herring stocks reported in the literature. Thus, the *A. simplex* (*s. s.*) population in herring from the Norwegian Sea appears to be the most differentiated one, whereas lowest level of genetic differentiation was observed between the North Sea and Baltic Sea populations. The results suggest that mtDNA *cox2* is a suitable genetic marker for *A. simplex* (*s. s.*) population structure analysis, which may also prove useful in further investigations of the herring stock structure in the NE Atlantic Ocean (see Mattiucci et al., 2017b).

3.1.4. Sardine (Sardina pilchardus)

Sardines from the Atlantic fishing ground (ICES IXa) showed comparatively high *Anisakis* spp. prevalence in both viscera (approximately 62%) and in the flesh (17%) which appears to be considerably higher

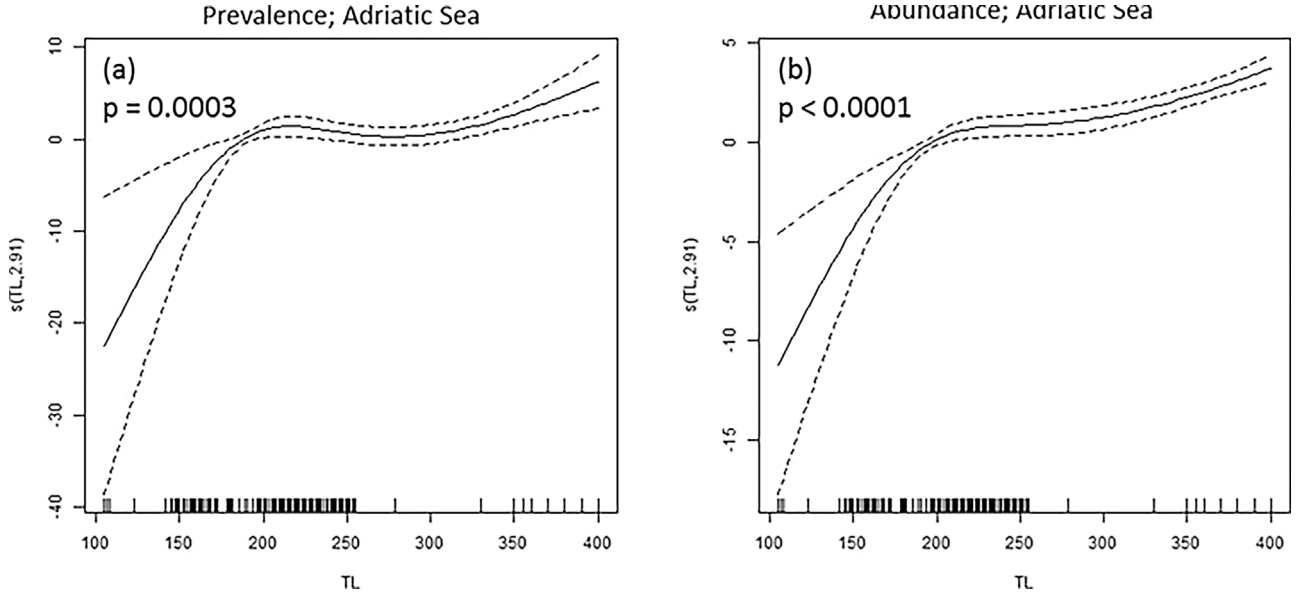

Fig. 5. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence (a) and abundance (b) of *Anisakis* spp. larvae in the flesh of chub mackerel (*Scomber colias*) from the Adriatic Sea. Dashed lines represent 95% conf. intervals around the main effects.

Table 3
Sample size and basic fish host biometric data, along with basic *A. simplex* (*s. s.*) infection parameters of herring (*Clupea harengus*) of four NE Atlantic stocks.

Herring stock	N fish	TL	TW	Musculature				Viscera	
				P (%)	Abundance/Intensity	Rel. distr. Vtrl: Drsl	Rel. distr.Left: Right	P (%)	Abundance/Intensity
North Sea	1252	273 ± 28 (149–358)	171 ± 55 (40–435)	17.4	A: 0.3 ± 0.7 (0–5) I: 1.6 ± 0.9 (1–5)	89: 11	50: 50	81.2	A: 11.2 ± 17.0 (0–176) I: 13.7 ± 17.6 (1–176)
Norw. spring spawning	726	334 ± 27 (275–405)	317 ± 70 (153–509)	37.1	A: 0.6 ± 1.0 (0–8) I: 1.6 ± 1.1 (1–8)	91: 9	51: 49	92.6	A: 11.2 ± 12.6 (0–112) I: 12.1 ± 12.7 (1–112)
Baltic	695	261 ± 29 (168–305)	153 ± 50 (31–268)	14.80	A: 0.2 ± 0.5 (0–3)	87: 13	54: 46	65.5	A: 4.2 ± 6.5 (0–45)
-Western	−600	216 ± 10 (189–240)	106 ± 9 (90–126)		I: 1.3 ± 0.5 (1–3)	Negative	Negative	0	I: 6.4 ± 7.1 (1–45)
-Central	−95				Negative				Negative

Abbreviations: TL – Total body length (mm); TW – Total body weight (g); P – Prevalence; A – Abundance, I – Intensity, both given as mean ± SD (range); Rel. distr. – Relative distribution (%); Vtrl – ventral portion of fish flesh (corresponds roughly to belly flap); Drsl – dorsal portion of fish flesh.

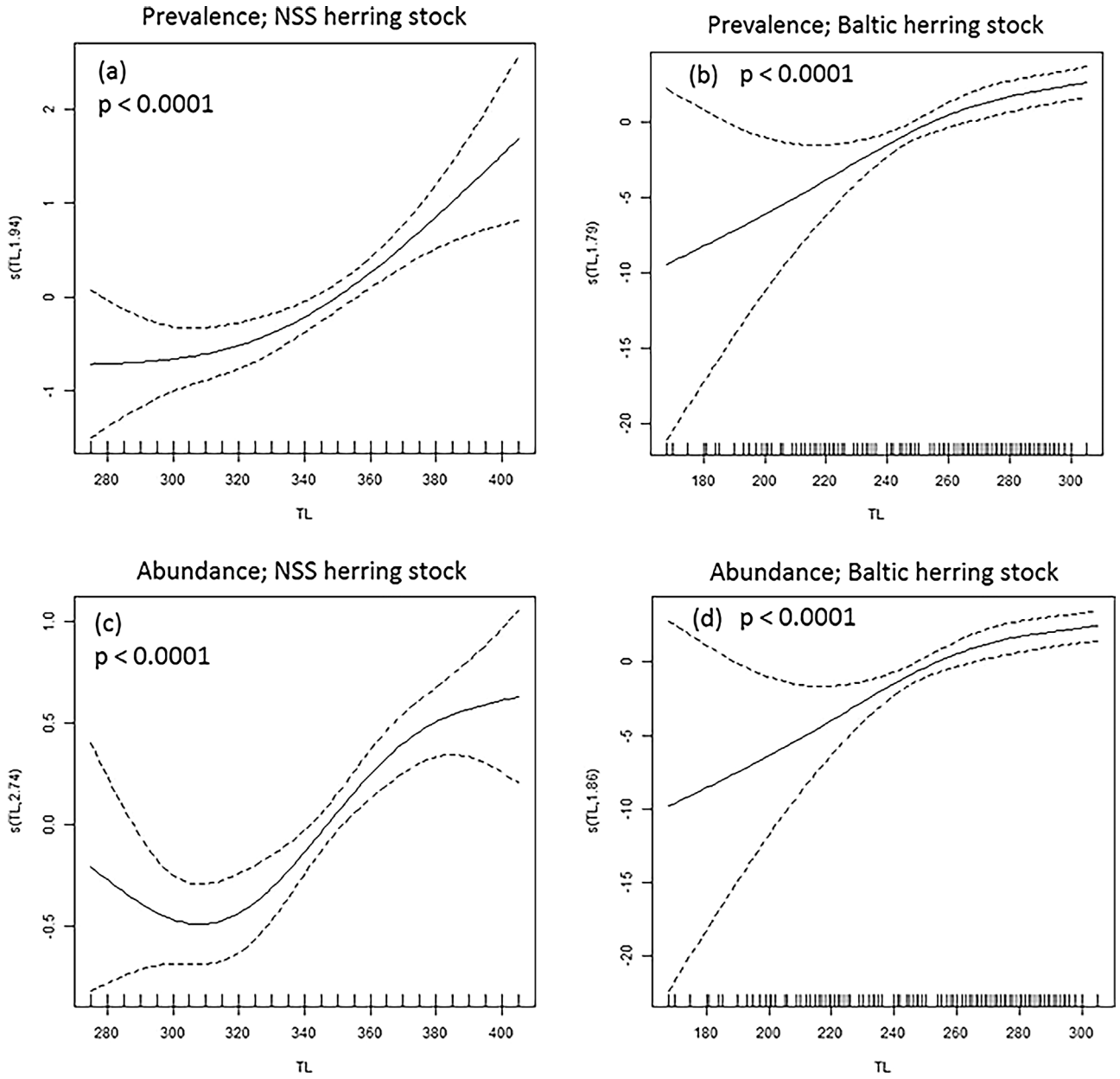

Fig. 6. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence and abundance of *A. simplex* (s. s.) larvae in the flesh of herring (*Clupea harengus*) belonging to the Norwegian spring spawning (NSS) (a, c) and Baltic (b, d) stocks. Dashed lines represent 95% conf. intervals around the main effects.

than previously reported infection levels in sardine from this fishing area (see Rodriguez et al., 2017).

In sardines from the Mediterranean, one of the most important findings is the occurrence of *A. pegreffii* in almost 50% of the fish caught off West Sardinia (FAO 37.1.1), exceeding all other Mediterranean locations. This finding is apparently closely related to fish host body size since sardines from the latter locality were on average more than 20 mm larger than the rest of the sample (Table 4). In contrast, the smallest of the present sardines, sampled in the northern Tyrrhenian Sea (FAO 37.1.3), were apparently free from *A. pegreffii* larvae. The significantly positive effect of host body size/length as main predictor of larval infection in the flesh of sardine from the present Mediterranean localities, is illustrated in Fig. 7.

Another important finding is the presence of larvae in sardine fillets which was observed for the first time in the present study. Consequently, the risk for consumers arising from these findings is obvious since sardines are often subjected to inadequate thermal processing or evisceration due to small fish size prior to preparation of marinated or salted home-made sardines, traditionally eaten throughout the Mediterranean region. Nonetheless, even in cases of higher prevalence, mean intensity of infection in sardines was usually low, between 1 and 2, both in fillets and viscera.

3.1.5. Anchovy (Engraulis encrasicolus)

There appears to be much higher *Anisakis* sp. prevalence and abundance in anchovies from central areas of the Adriatic Sea and off Galicia (Spain) which makes anchovy a food safety-related "hotspot" in parts of Italy and Spain. For the Atlantic samples, *Anisakis* spp. prevalence reached 83% and 29% in the viscera and flesh, respectively, in some batches (Rodriguez et al., 2017). Similar high infection levels were reported from the Central Adriatic Sea, with prevalences reaching 69.5% and 14.6% in the viscera and flesh, respectively (Cipriani et al., 2017a). In either catching area, fish host body size was a significant predictor of larval prevalence and abundance in the fish flesh, although the former relation was only weak for anchovy from the Central Adriatic Sea (Fig. 8).

All larvae (N = 547) obtained from anchovies caught in the Mediterranean Sea, correspond to *A. pegreffii*. The infection levels of *A. pegreffii* were significantly different between the present fishing areas of the Mediterranean Sea. Thus, fish from the Central and South Adriatic Sea showed highest prevalence and intensity, while anchovies from off southern Sicily and the Ionian- and Alboran Sea were apparently uninfected. The vast majority (95.8%) of *A. pegreffii* larvae were located in the body cavity while only a smaller fraction (4.2%) was present in the fish flesh. According to the present infection data for *A. pegreffii* in *E. encrasicolus*, the Central Adriatic Sea appears to be a "hotspot" for the presence of this parasite, showing infection levels which are by far higher than in any other area of the Mediterranean Sea. This finding could thus be related to ecological or oceanographic characteristics of this basin, including both abiotic and biotic factors which contribute to maintain the life cycle of *A. pegreffii* at high population size (Cipriani et al., 2017a).

3.1.6. Atlantic cod (Gadus morhua)

There were considerable differences in *Anisakis* spp. infection level in cod from different sampling areas, e.g. the prevalence varied between 16% in Baltic cod and 100% in cod from the Barents Sea. In general, *Anisakis* spp. prevalence and abundance, both in whole fish and in the fish flesh, correlated with fish length, and tended to increase with increasing fish size. Fishing area appeared to be a significant effector of *Anisakis* spp. infection in the fillets, with higher prevalence for fish sampled in the Barents Sea compared to the North Sea and the Baltic Sea. However, cod from the Baltic Sea were significantly smaller than the other fish sampled. In the North Sea area, two ICES subdivisions were sampled, i.e. the northern North Sea (IVa) and the Central North Sea (IVb). Even though these samples have been grouped for the statistical analysis (see Gay et al., 2017), the prevalence both in fillets and overall fish were statistically different for these two sub-areas ($p < 0.05$). The infection pattern in area IVa was similar to the pattern seen in cod from the Barents Sea compared to area IVb. In the flesh of cod, most larvae resided in the ventral portion of the fillets, which in cod corresponds largely to the belly flaps. In pooled samples covering all sampling areas, the prevalence of *Anisakis* spp. in the belly flaps was 39% while only 12% carried larvae in the dorsal part of the fillets.

Other zoonotic ascaridoid genera were also observed. *Pseudoterranova* was present in the flesh of 12% of the sampled fish, with a peak of prevalence of 27% for the northern North Sea sample. *Hysterothylacium* prevalence varied between 0% for the Baltic Sea and 84% for the Barents Sea. The distribution of *Contracaecum* was also very variable, with no *Contracaecum* isolated from the fish from the Central North Sea and a prevalence of 100% for the commercial size cod sampled in the Baltic Sea (see Gay et al., 2017).

3.1.7. Haddock (Melanogrammus aeglefinus)

Haddock were sampled around Scotland (East and West coasts) and in the Barents Sea. Average length was highest in the Barents Sea (584 mm) and higher on the East coast than on the West coast of Scotland (365 mm vs 311 mm). Indeed, there was almost no overlap in size between Scottish and Barents Sea fish. Prevalence and abundance of *Anisakis* sp. (genetic species identification was not performed) infection followed the same trend as average size: 50% prevalence and average abundance 3.4 on the West coast of Scotland, compared to 84% and 14.5 on the East coast, and 100% and 50.5 in the Barents Sea. Highest *Anisakis* sp. intensity was 183 in a haddock from the Barents Sea. Prevalence and abundance of larvae increased significantly with fish size in the present samples ($p < 0.001$ in both cases). In Scottish

Table 4
Sample size and basic fish host biometric data, along with basic *Anisakis pegreffii* infection parameters of sardine (*Sardina pilchardus*) from four Mediterranean sampling localities.

Fishing area	N fish	TL	TW	Musculature				Viscera	
				P (%)	Abund./Intensity	Rel. distr.Vtrl: Drsl	Rel. distr. Left:Right	P (%)	Abund./Intensity
Overall Adriatic Sea sample (FAO 37.2.1)	908	137 ± 8 (97–180)	20 ± 5 (6–45)	0.8	A: 0.01 ± 0.1 (0–2) I: 1.1 ± 0.4 (1–2)	75:25	25:75	2.9	A: 0.05 ± 0.4 (0–10) I: 1.8 ± 1.9 (1–10)
Northern Tyrrhenian Sea (FAO 37.1.3)	100	130 ± 6 (115–145)	13 ± 3 (10–21)	0	/	/	/	0	/
South Sicily (FAO 37.2.2)	200	140 ± 8 (120–170)	25 ± 4 (16–40)	0	/	/	/	1.5	A: 0.03 ± 0.23 (0–3) I: 1.67 ± 1.15 (1–3)
West Sardinia (FAO 37.1.1)	356	173 ± 12 (140–200)	40 ± 8 (18–58)	4.8	A: 0.05 ± 0.3 (0–2) I: 1.1 ± 0.3 (1–2)	89: 11	32: 68	42.4	A: 0.7 ± 1.1 (0–7) I: 1.7 ± 1.1 (1–7)

Abbreviations: TL – Total body length (mm); TW – Total body weight (g); P – prevalence (%), A – Abundance given as mean ± SD (range); I – Intensity given as mean ± SD (range); Rel. distr. – Relative distribution (%); Vtrl – ventral portion of fish flesh (corresponds roughly to belly flap); Drsl – dorsal portion of fish flesh.

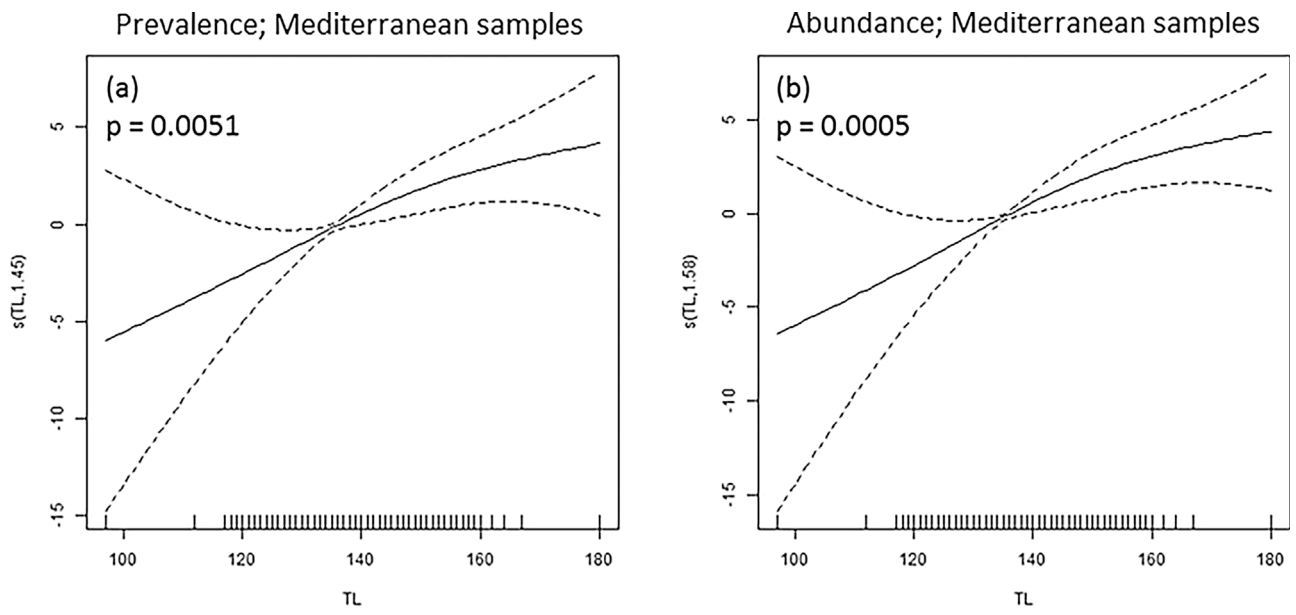

Fig. 7. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence (a) and abundance (b) of *Anisakis* spp. larvae in the flesh of sardine (*Sardina pilchardus*) from Mediterranean fishing areas. Dashed lines represent 95% conf. intervals around the main effects.

fish, *Anisakis* sp. were present in 1% of fillets from West coast fish and 11% of fillets from East coast fish, prevalence in fillets was much higher in Barents Sea fish, at 73%, although the mean number present was < 1 and the maximum only 12.

Pseudoterranova sp., *Hysterothylacium aduncum* and *Contracaecum* sp. were all recorded, with higher prevalence and abundance in Barents Sea fish. Of these, *Contracaecum* sp. was most prevalent (36%) but occurred at lowest abundance (mean 0.6, maximum 7), *H. aduncum* had the highest abundance (mean 1.8, maximum 62) while *Contracaecum* sp. was not recorded in Scottish fish but again *H. aduncum* was the most abundant of the three genera (mean 0.9 and maximum 13 in East coast samples) (see also Pierce et al., 2017).

3.1.8. European hake (Merluccius merluccius)

Both prevalence and abundance of *Anisakis* spp. infection were very high in hake caught in the Grand Sole Bank area, the Cantabric Sea and off NW Spain and Portugal (ICES VIIj, VIIIc and IXa). Highest larval infection levels were found in fish from the Grand Sole Bank (ICES VIIj), with mean and maximum abundance in the flesh reaching 129 and 1484 larvae, respectively. These were the highest infection levels recorded in the present survey of the PARASITE project, regardless of fish host species or geographical sampling area. Due to the importance of hake for various European fresh fish markets, we consider such *Anisakis* spp. infection levels in the fish flesh as very high, which again calls for proper precautionary heat- or freezing treatment before consumption.

Mixed infections with *A. simplex* (*s. s.*) and *A. pegreffii* were recorded in subsamples of larvae (n = 2806) from hake caught off NW Spain and Portugal, with the majority (ca 70%) genetically identified as *A. simplex* (*s. s.*) while approx. 30% belonged to *A. pegreffii*. The findings confirm that this particular fishing ground represents a marked sympatric area for both parasite species. GAM analysis revealed that *Anisakis* spp. prevalence and abundance in the flesh increased with host body size/length, exemplified in Fig. 9 (b) for effect on larval abundance in hake from the Grand Sole Bank, which showed 100% larval prevalence in the flesh. However, some variations of this general trend exist depending on Atlantic fishing area, which is addressed in more detail in Pascual et al. (2017).

Generally, all samples obtained from the fishing grounds of the western Mediterranean Sea showed comprehensively lower infection levels when compared to the fish originating from the Adriatic/Ionian Sea area. Indeed, highest infection levels (total prevalence (100%) and overall mean abundance A = 157.3, ranging 4–866) were recorded in hake caught off the Italian coast of the southern Adriatic Sea. Moreover, fish from the latter area were significantly larger (mean length 416 mm) compared to fish from other parts of the Adriatic Sea ($p < 0.001$). High infection levels have been recorded also in hake caught off the Italian coast of the central Adriatic Sea (prevalence P = 89.8% and mean abundance A = 98.9), despite of the on average smaller size of the fish obtained from this area (Cipriani et al., 2017b). Thus, a significantly positive relationship exists between fish host size/length and *Anisakis* spp. prevalence and abundance in the flesh of hake in pooled samples (n = 466) from the present Adriatic fishing localities (Fig. 9 a, c). This implies that fish host body size is the major driver of *Anisakis* spp. occurrence and infection level in hake from both Atlantic and Mediterranean fishing grounds.

Among the *Anisakis* spp. subsamples genetically identified from Mediterranean hake, close to 93% corresponded to *A. pegreffii,* 1 larva to *A. simplex* (*s. s.*) and 62 to *A. physeteris*. While *A. pegreffii* occurred around the viscera and in the flesh of the hake, *A. physeteris* larvae were only found in the visceral cavity.

3.1.9. Blue whiting (Micromesistius poutassou)

Blue whiting from Rockall (ICES area VIb) showed high prevalence and abundance of *Anisakis* larvae, both with respect to overall infection and infection in the fish flesh. Larval muscle infection reached a mean abundance of more than 14 larvae at > 92% prevalence while the maximum larval intensity in the flesh was 147 worms. In contrast, blue whiting sampled in the other NE Atlantic fishing areas including waters around Scotland (ICES IVa and VIa) and the Faroe Islands (ICES Vb), and off Portugal and NW Spain (ICES VIII–IX), showed significantly lower infection rates, with larval prevalence and mean abundance in the fish flesh reaching 52% and 6.1, respectively (Table 5). The maximum intensity was 69 larvae, which implies that the infection rates in the latter samples must still be considered high. However, blue whiting from the Rockall area were significantly larger than those from the other Atlantic areas ($p < 0.001$). For the Atlantic blue whiting samples in general, there was a significantly positive correlation between fish host size (TL) and larval abundance, both in total and in the flesh ($p < 0.001$ for both infection sites). However, this trend weakened with lower latitudes, i.e. in the blue whiting samples from off NW Spain and Portugal, larval abundance was only weakly ($p < 0.02$) correlated with fish host length.

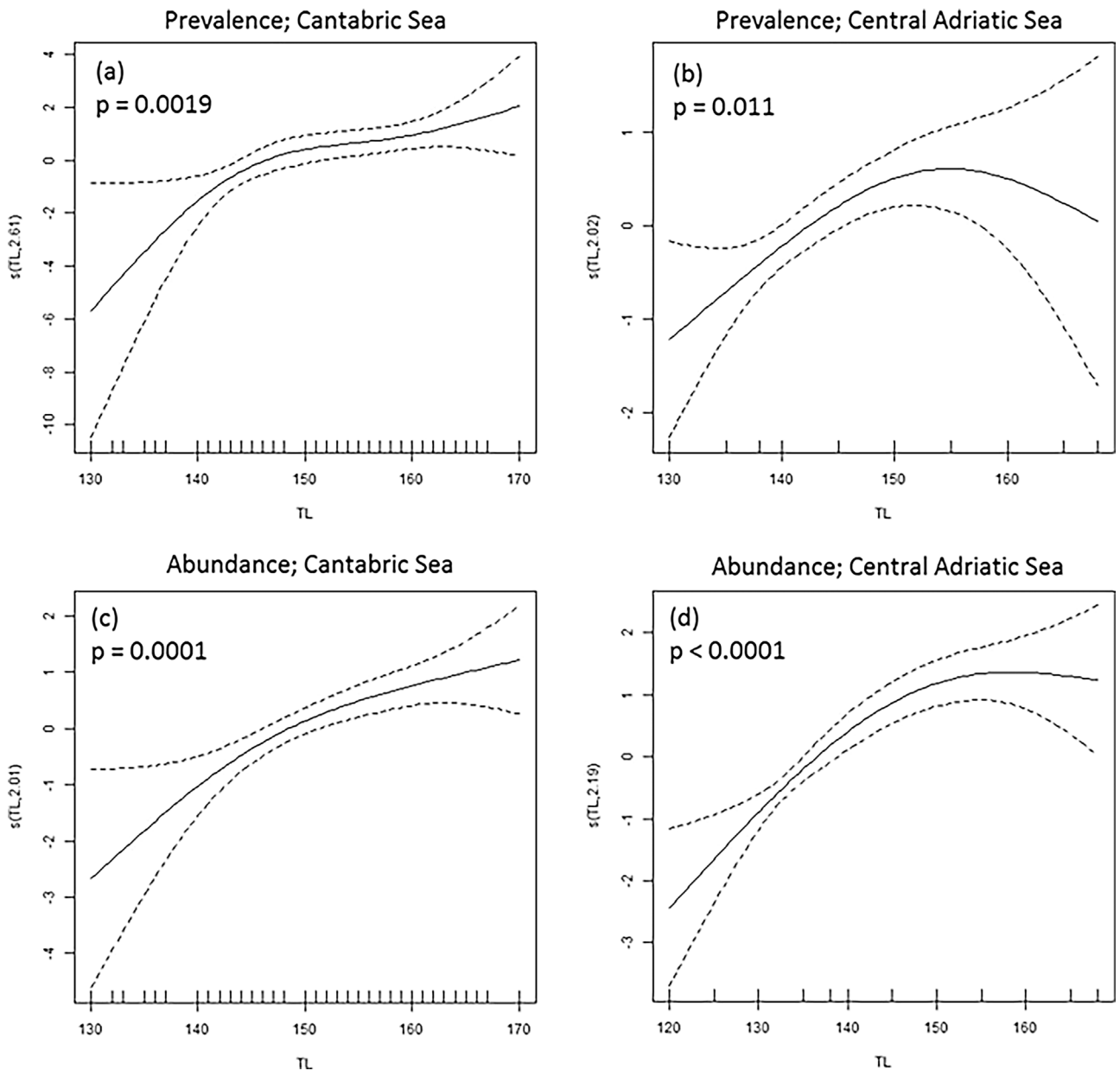

Fig. 8. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence and abundance of *Anisakis* spp. larvae in the flesh of anchovy (*Engraulis encrasicolus*) from the Cantabric Sea (a, c) and the Central Adriatic larval infection "hotspot" (b, d). Dashed lines represent 95% conf. intervals around the main effects.

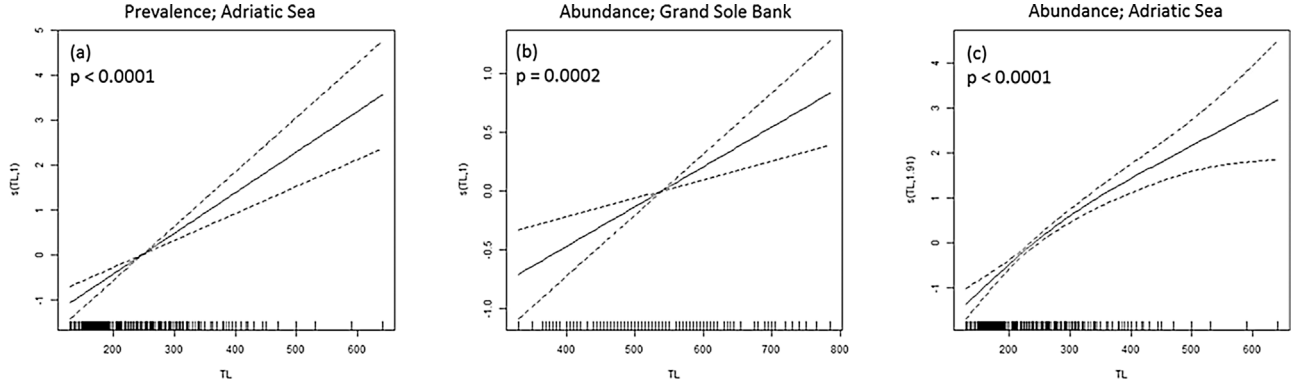

Fig. 9. GAM smoothing curves fitted to effect of fish host body length (TL) on prevalence and abundance of *Anisakis* spp. larvae in the flesh of European hake (*Merluccius merluccius*) from the Grand Sole Bank (b) and the Adriatic Sea (a, c). Dashed lines represent 95% conf. intervals around the main effects.

Blue whiting sampled in the Mediterranean showed generally much lower *Anisakis* larval infection levels compared to their congeners from the Atlantic fishing areas. However, blue whiting from the Adriatic Sea still reached comparatively high overall infection values, presenting a maximum intensity of 305 and 8 worms in overall and muscle infection, respectively, at 34% prevalence of larvae residing in the flesh. In contrast, fish from the Alboran Sea showed only low values of *Anisakis*, e.g. very low prevalence and abundance in the fish flesh (3% and 0.04, respectively), with maximum intensity not exceeding two larvae (Table 5). Although blue whiting from the Adriatic Sea represented by far the smallest fish examined, they still carried significantly higher *Anisakis* burden than their congeners from the Alboran Sea ($p < 0.001$).

Genetic species identification revealed that the subsample of 124 *Anisakis* larvae from blue whiting caught in the Norwegian Sea and off the Faroe Islands consisted of *A. simplex* (*s. s.*). A subsample of 37 worms from off the Portuguese coast (ICES IX) showed a mixed infection with *A. simplex* (*s. s.*) (30 specimens) and *A. pegreffii* (7 specimens). The whole subsample of 134 worms from *M. poutassou* fished in the Adriatic Sea consisted entirely of *A. pegreffii*.

3.1.10. Whiting (Merlangius merlangus)

Whiting were sampled from trawling surveys on the East and West coasts of Scotland (ICES areas IV and VI, respectively). Fish from the West coast were larger on average, mainly due to the presence of larger numbers of fish < 120 mm in length that were almost absent from West coast samples. Prevalence and abundance of *Anisakis* sp. (genetic species identification was not performed) were significantly higher in larger fish ($p < 0.001$ in both cases). Overall prevalence of *Anisakis* sp. was slightly (but non-significantly) higher on the West coast (50.4% versus 42.6%, $p = 0.08$), although mean abundance was slightly (again non-significantly) higher in East coast fish (7.9 versus 6.4). Abundance of *Anisakis* sp. in the fillets was higher in the East coast samples (22.7% versus 8.9%, $p < 0.001$), with high numbers present in fillets of larger East coast fish but not in larger West coast fish. *Hysterothylacium* was recorded in 12.0% of West coast fish and 19.9% of East coast fish while *Pseudoterranova* was present in 1.0% and 2.7% of fish, respectively (see also Pierce et al., 2017).

3.1.11. Plaice (Pleuronectes platessa) and four-spotted megrim (Lepidorhombus boscii)

For pooled samples, i.e. all geographical areas included, four-spotted megrim displayed higher *Anisakis* spp. prevalence in overall fish, in fillets and in viscera (60.6; 30 and 51.9%, respectively) than plaice (23.9; 0.7 and 23.7%, respectively) (Table 6). Mean weight and length of all batches from both species were quite similar, so that the differences were likely not related to fish size. Thus, the differences could be due to geographical origins since both species were sampled in different ICES areas (North Sea, West of Scotland and English Channel for the plaice, and Bay of Biscay, Portuguese waters and Southwest of Ireland for the four-spotted megrim). In each species, geographical area induced significant differences. Highest prevalence (42.7%) was observed in plaice sampled in the West of Scotland, while highest prevalence in four-spotted megrim was observed for fish sampled in the Southwest of Ireland (82.1%). The highest intensities were observed in four-spotted megrim sampled in the southwest of Ireland, reaching maximum 80 larvae. In plaice, highest abundance was observed off West Scotland with values up to 28. Larval prevalence in the flesh of plaice was very low, with only 3 infected fish out of 464 sampled (0.7%), whereas almost one third (30%) of the four-spotted megrim carried *Anisakis* spp. larvae in the flesh. A subsample of 11 larvae isolated from plaice caught in the English Channel (ICES VIId) were molecularly identified and were all assigned to *Anisakis simplex* (*s. s.*).

3.1.12. Monkfish and black-bellied angler (Lophius piscatorius and L. budegassa)

The two *Lophius* species were sampled in relatively small numbers. Monkfish was obtained from Atlantic waters, ICES areas VII, VIII and IX, as well as from the Alboran Sea of the Mediterranean, while black-bellied angler was also sampled in the Balearic- and Adriatic Seas. Highest overall *Anisakis* spp. prevalence and abundances were seen in areas VII and VIIIc, respectively, apparently with a slight tendency of small monkfish to show higher larval abundances in the flesh. *Anisakis* spp. abundance (but not prevalence) generally increased with fish length and differences were observed between different sampling periods. A difference in *Anisakis* spp. prevalence was found between male and female *L. budegassa*, with a higher prevalence of worms in female fish. It is worth noticing that the samples of monkfish caught in the Alboran Sea showed mixed infections with three *Anisakis* species, i.e. *A. simplex* (*s. s.*), *A. pegreffii* and *A. physeteris*, identified by allozymes and

Table 5
Sample size and basic fish host biometric data, along with basic *Anisakis* spp. infection parameters of blue whiting (*Micromesistius pouttassou*) from six different fishing areas.

Sampling area (ICES zone)	N fish N = 1534	TL	TW	Musculature				Viscera	
				P (%)	Abundance/Intensity	Rel. distr. Vtrl: Drsl	Rel. distr. Left: Right	P (%)	Abundance/Intensity
North Sea, Off W Scotland (ICES IVa, VIa)	153	223 ± 43 (96–333)	68 ± 41 (5–255)	27.6	A: 0.5 ± 1.1 (0–6) I: 1.8 ± 1.4 (1–6)	93: 7	55: 45	71.1	A: 7.0 ± 10.9 (0–77) I: 9.9 ± 11.7 (1–77)
Off Faroe Isles (ICES Vb)	300	278 ± 30 (182–352)	119 ± 36 (33–285)	52.3	A: 6.1 ± 11.8 (0–69) I: 11.7 ± 14.2 (1–69)	89: 11	56: 44	89.0	A: 15.7 ± 23.9 (0–165) I: 16.8 ± 23.3 (1–165)
Rockall area (ICES VIb)	454	324 ± 150 (225–455)	171 ± 77 (56–558)	93.0	A: 14.1 ± 18.5 (0–147) I: 15.2 ± 18.8 (1–147)	90: 10	46: 54	98.5	A: 36.4 ± 42.4 (0–309) I: 37.0 ± 42.5 (1–309)
Off Spain & Port. (ICES VIIIc, IX)	386	255 ± 34 (200–380)	104 ± 47 (38–382)	50.5	A: 2.1 ± 5.6 (0–45) I: 4.1 ± 7.3 (1–45)	96: 4	52: 48	96.9	A: 24.1 ± 70.9 (0–649) I: 24.9 ± 71.9 (1–649)
Mediterranean Sea	241	251 ± 16 (220–295)	118 ± 24 (81–191)	2.9	A: 0.04 ± 0.3 (0–2)	/	/	1.4	A: 0.01 ± 0.1 (0–1)
Alboran Sea	69	198 ± 31 (111–312)	64 ± 31 (8–229)	34.1	I: 1.5 ± 0.7 (1, 2)	96: 4	49: 51	85.0	I: 1 ± 0 (1)
Adriatic Sea	172				A: 0.8 ± 1.5 (0–8) I: 2.3 ± 1.8 (1–8)				A: 36.3 ± 61.8 (0–303) I: 42.7 ± 65.0 (1–303)

Abbreviations: TL – Total body length (mm); TW – Total body weight (g); P – Prevalence; A – Abundance, I – Intensity, both given as mean ± SD (range); Rel. distr. – Relative distribution (%); Vtrl – ventral portion of fish flesh; Drsl – dorsal portion of fish flesh.

Table 6
Sample size and basic fish host biometric data, along with basic *Anisakis* infection parameters of plaice (Pp, *Pleuronectes platessa*) and four-spotted megrim (Lb, *Lepidorhombus boscii*) from eight North East Atlantic sampling localities.

Fishing area	N fish (species)	TL	TW	Musculature				Viscera	
				P (%)	Abund./Intensity	Rel. distr. Vtrl: Drsl	Rel. distr. Left: Right	P (%)	Abund./Intensity
Southern North Sea (ICES IVc)	56 (Pp)	285 ± 25 (237–351)	244 ± 76 (130–491)	0.0	/	/	/	5.4	A: 0.09 ± 0.39 (0–2) I: 1.67 ± 0.58 (1–2)
English channel (ICES VIId)	184 (Pp)	302 ± 52 (210–570)	300 ± 236 (98–1907)	0.5	A: 0.01 ± 0.07 (0–1) I: 1.00 ± 0.00 (1-1)	f.p.	f.p.	12.5	A: 0.15 ± 0.42 (0–3) I: 1.17 ± 0.49 (1–3)
Northern North Sea (ICES IVa)	12 (Pp)	297 ± 45 (196–365)	262 ± 105 (70–475)	0.0	/	/	/	33.3	A: 3.25 ± 6.69 (0–23) I: 9.75 ± 8.92 (4–23)
Central North Sea (ICES IVb)	102 (Pp)	294 ± 42 (220–384)	265 ± 116 (91–540)	1.0	A: 0.02 ± 0.20 (0–2) I: 2.00 ± 0.00 (2-2)	f.p.	f.p.	32.4	A: 0.86 ± 1.66 (0–8) I: 2.67 ± 1.93 (1–8)
West of Scotland (ICES VIa)	110 (Pp)	257 ± 38 (220–271)	143 ± 84 (91–209)	0.9	A: 0.01 ± 0.10 (0–1) I: 1.00 ± 0.00 (1-1)	f.p.	f.p.	42.7	A: 2.94 ± 5.28 (0–28) I: 6.87 ± 6.20 (1–28)
Portuguese waters (ICES IX)	48 (Lb)	250 ± 19 (200–292)	125 ± 30 (66–201)	12.5	A: 0.13 ± 0.33 (0–1) I: 1.00 ± 0.00 (1-1)	f.p.	f.p.	45.8	A: 0.65 ± 0.86 (0–4) I: 1.41 ± 0.73 (1–4)
Southwest of Ireland – East (ICES VIIj)	67 (Lb)	286 ± 31 (230–350)	183 ± 70 (73–411)	65.7	A: 2.19 ± 3.04 (0–16) I: 3.34 ± 3.20 (1–16)	99: 1	56: 44	82.1	A: 8.61 ± 11.35 (0–75) I: 10.49 ± 11.73 (1–75)
Southwest of Ireland – West (ICES VIIk)	72 (Lb)	319 ± 33 (265–485)	232 ± 88 (133–771)	33.3	A: 1.32 ± 4.12 (0–28) I: 3.96 ± 6.45 (1–28)	94: 6	52: 48	36.1	A: 1.50 ± 3.50 (0–19) I: 4.15 ± 4.83 (1–19)
Bay of Biscay (ICES VIII)	100 (Lb)	243 ± 24 (200–315)	112 ± 37 (62–232)	12.0	A: 0.17 ± 0.49 (0–2) I: 1.42 ± 0.51 (1–2)	88: 12	29: 71	46.0	A: 1.36 ± 3.05 (0–21) I: 2.96 ± 3.96 (1–21)

Abbreviations: TL – Total body length (mm); TW – Total body weight (g); P – prevalence (%), A – Abundance, I – Intensity, both given as mean ± SD (range); Rel. distr. – Relative distribution (%); Vtrl – ventral portion of fish flesh (corresponds roughly to belly flap); Drsl – dorsal portion of fish flesh; f.p. – too few parasites for appropriate calculation.

mtDNA cox2 sequence analysis. The actual larvae occurred in syntopy in the viscera of the same individual fish host.

3.1.13. Silver scabbardfish (*Lepidopus caudatus*) and European sea bass (*Dicentrarchus labrax*)

Silver scabbardfish were obtained from four sampling areas of the Mediterranean Sea (Table 1). All fish regardless of sampling area were infected with *Anisakis* spp. larvae, i.e. P = 100%. Scabbardfish caught off Malta's coast and from off southern Sicily showed significantly higher overall larval abundance levels (MA = 239 ± 141 and MA = 193 ± 118, respectively) compared to the fish from the other sampling areas (MA = 50 ± 59 in pooled samples) ($p < 0.001$). However, and more importantly, the larval abundance in the fish muscle did not differ significantly between the different sampling areas, showing 47% prevalence and MA = 2.2 ± 4.0 with intensity ranging 1–24 in pooled fish samples. All genetically identified larvae from the former areas belonged to *A. pegreffii*. Identification of larvae from scabbard fish sampled in the Alboran- and Tyrrhenian Seas revealed that *A. pegreffii* was still the most abundant sibling species, however, a few *A. physeteris* larvae (N = 25) were found in both areas, occurring in syntopy with *A. pegreffii* in the viscera of one individual fish host in either locality. Interestingly, in the fish batch originating from the Alboran Sea, a single scabbard fish had a mixed infection with *A. simplex* (*s. s.*) (1 larva), *A. nascettii* (1 larva), and *A. ziphidarum* (1 larva), along with a majority of larvae belonging to *A. pegreffii*.

Parasite data of European sea bass are scarce. Of 38 fish collected and examined during the present survey, only two medium-sized specimens weighing 480 and 500 g, and fished in the central Tyrrhenian Sea, appeared to be infected with 12 and 1 anisakid larvae, respectively, all situated around the organs of the visceral cavity. Sequence analysis of the mtDNA *cox2* gene enabled to identify the nematodes as *Contracaecum rudolphii* sp. A, which matures and reproduces in piscivorous birds (Mattiucci et al., 2008).

3.2. *Anisakis* spp. exposure assessment considerations

3.2.1. Effect of fish sampling and storage procedures

The different post-catch storage modes applied by the surveyors before inspecting the fish for parasites, may under some circumstances have facilitated post-mortem migration of *Anisakis* spp. larvae from their original site around the visceral organs into the flesh of the hosts. While this is unlikely to occur in fish that is deep-frozen or eviscerated shortly after catch, larval post-mortem migration may take place in cases where the fish is cool-stored for several hours during transport and before processing. Such storage conditions may have prevailed in at least some of the present Mediterranean samples of anchovy and sardine obtained from local fishermen. Larval muscle penetrating behavior may also be facilitated by the very short migration distance between the visceral organs and the flesh of small fish such as anchovy and sardine. Thus, temperature and storage time appear to be the most important variables determining the activation and motility of *Anisakis* larvae, as observed in experimental studies (Cipriani et al., 2016; Šimat et al., 2015). Although storage temperatures above 2 °C seem to be required to induce any post-mortem migration of *A. pegreffii* larvae in anchovy (Cipriani et al., 2016), higher storage temperatures over shorter periods may have been the case for some of the present anchovy and sardine samples from the Mediterranean Sea. Thus, the possibility exists that at least some of the larval findings in the flesh of both anchovy and sardine may have been inflicted by post-mortem migration. Nevertheless, cool-storage and sales of freshly caught, i.e. unfrozen, anchovy and sardine illustrates the importance of these fish species as vector of anisakiasis when prepared raw or only lightly processed in private households in actual regions of Italy and Croatia (Moschella et al., 2004; Mattiucci et al., 2011, 2013; Mladineo et al., 2016).

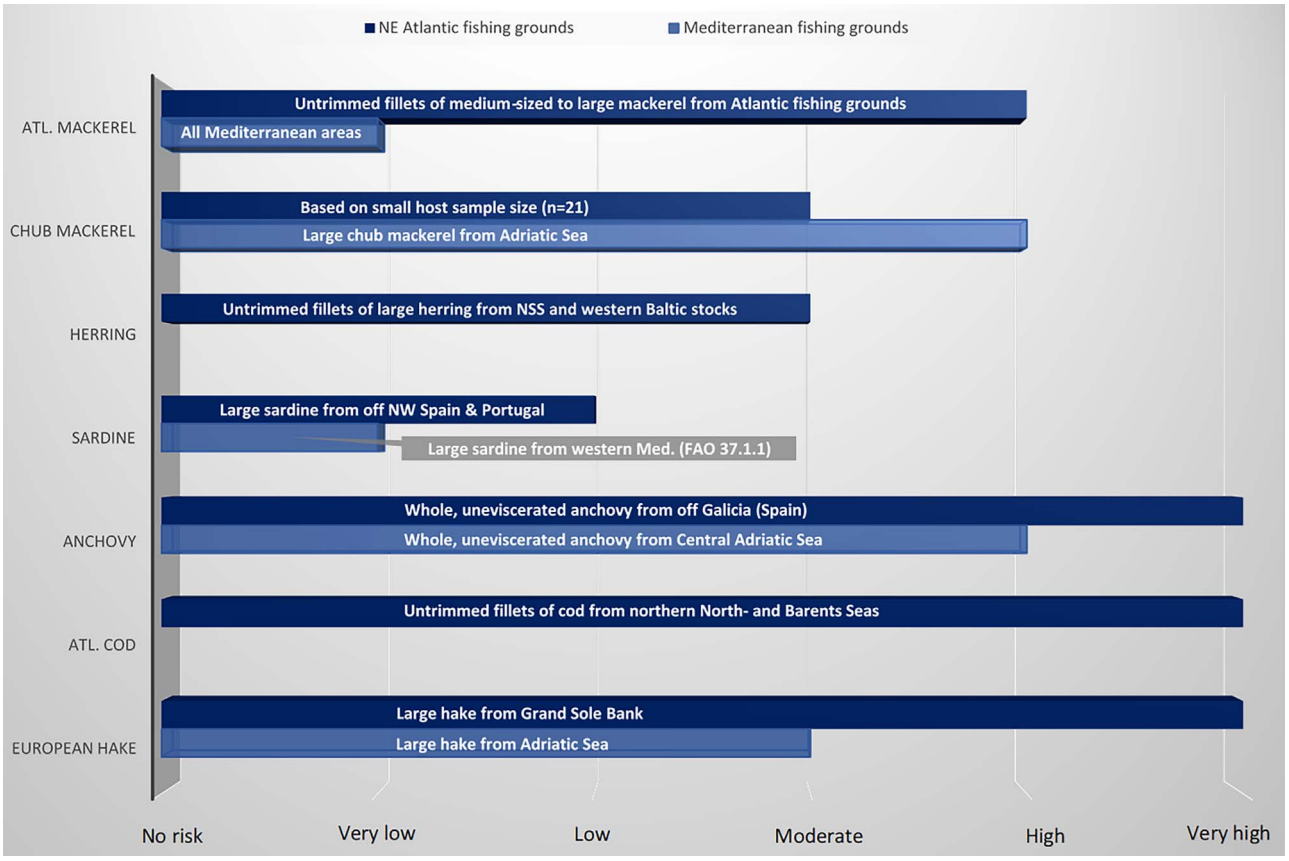

Fig. 10. *Anisakis* spp. exposure risk profile based on parasite prevalence in the flesh of actual fish species if to be consumed raw (sushi/sashimi), or only lightly processed (pickled/marinated/cold-smoked). The risk categories reflect the following *Anisakis* spp. prevalence ranges in the fish flesh: No risk: 0%; Very low: 1–5%; Low: 6–20%; Moderate: 21–50%; High: 51–80%; Very high: > 80%.

3.2.2. *Effect of fish host*

Results from GAM analyses indicated that fish host body size is a major predictor of *Anisakis* spp. occurrence in most fish species presently analyzed. Thus, there was a clear trend towards significantly positive relationships between fish host body size/length and both prevalence and abundance of *Anisakis* spp. larvae in the fish flesh. However, exceptions from this trend became apparent in Atlantic mackerel where the host length effect on larval muscular infection was only very weakly positive or even absent in the present southern- or northernmost fishing localities, respectively. These findings indicate that any accumulation over time of *Anisakis* spp. larvae does not take place in Atlantic mackerel. On the contrary, a trend towards the opposite pattern, i.e. larger mackerel are carrying fewer larvae, at least in some of the present Atlantic localities, could indicate that larger specimens of the actual stocks or entities are able to cope with the infections immunologically. These aspects of the *Anisakis* spp. infection pattern in the present Atlantic mackerel samples are addressed and discussed in detail by Levsen et al. (2017). Besides mackerel, a less significant effect of host body size on *A. pegreffii* prevalence was apparently present in anchovy from certain Atlantic and Mediterranean fishing localities. Thus, other biotic or abiotic factors that are characteristics of the actual localities, may be important drivers of the observed infection patterns, as well. For details on these aspects of *Anisakis* spp. occurrence in anchovy from Atlantic and Mediterranean fishing grounds, see Rodriguez et al. (2017) and Cipriani et al. (2017a), respectively.

In general, the effect of body size on infection rate seems to be strongly related to an ontogenetic change in preferred food source by switching from microplanktonic food to larger prey including macroplankton such as krill, or to a mainly piscivorous feeding habit. This has been demonstrated for several fish species including herring and Atlantic cod (Levsen and Lunestad, 2010; Mouritsen et al., 2010; Münster et al., 2015) to greatly increase anisakid infection probability. This again may result in larval accumulation over time since *Anisakis* spp. larvae may stay alive for extended periods or even over a given fish host's lifetime (Smith, 1984). The observed distribution of anisakid larvae exhibited very high spatial and temporal variability, even within host species. Highly skewed distribution patterns are characteristic for many parasite species (e.g., Shaw and Dobson, 1995) and *Anisakis* species appear to be typical in this respect.

In the present herring samples we observed a highly significant increase in overall *Anisakis simplex* (*s. s.*) abundance with increasing fish size. Similarly, the extremely high *Anisakis* spp. infection levels in both flesh and viscera of European hake from the Grand Sole area (ICES VII) strongly suggest that also this case relates most likely to fish host body size since hake from this particular fishing ground were by far the largest fish (both in terms of TL and TW) of all hake examined in the present survey (for details, see Pascual et al., 2017).

The possible presence of *Anisakis* spp. larvae in the fish flesh represents the greatest concern with respect to consumer exposure risk, especially in fish intended for consumption in a fresh state, i.e. without prior freezing. Thus, the present survey revealed that the vast majority of the flesh residing larvae are situated in the ventral portion of the fish muscle, which in most fish species corresponds to the belly flaps. This trend was especially pronounced in the Atlantic samples of chub mackerel (Table 2), Norwegian spring spawning herring (Table 3), blue whiting (Table 5), and Atlantic cod (see Gay et al., 2017). Thus, trimming of the fillets by removing most of the belly flaps would significantly reduce the probability of *Anisakis* larvae to be present in the final product. For example, Levsen and Lunestad (2010) found when investigating the *Anisakis simplex* (*s. l.*) occurrence in Norwegian spring spawning herring, that the probability of larval presence in trimmed

herring fillets (by removing the belly-flaps) was 5–8 times lower compared to untrimmed fillets.

It is also apparent that, for all samples analyzed, larval mean abundances observed in the fish flesh were generally correlated with the number of worms found in the visceral cavity/organs (global analysis of mean abundances for all samples, $p < 0.001$). However, an exception from this general trend was seen in Atlantic mackerel where several specimens, up to 5.2% of each batch and sampling locality, carried *Anisakis* spp. larvae in the flesh only, i.e. the visceral cavity was not infected (see Levsen et al., 2017).

3.2.3. Effect of fishing ground/oceanographic area

The most conspicuous difference between the Atlantic- and Mediterranean fishing grounds with regards to general *Anisakis* spp. infection characteristics relates to the relative occurrence of *Anisakis* sibling species. Thus, *A. simplex* (*s. s.*) is the predominating species in the Atlantic samples of the present survey, while *A. pegreffii* dominates all samples from the Mediterranean Sea. However, there is considerable sympatric overlap between the western Mediterranean (Alboran Sea) and the Bay of Biscay in the Atlantic. Moreover, a few *A. pegreffii* were also found in Atlantic mackerel as far north as the Norwegian Sea. Since Atlantic mackerel, which spawn in deep water between the Mediterranean and the Faroe Islands, undertake extensive feeding migrations between the southern spawning- and the northern feeding grounds (Jansen and Gislason, 2013), the finding of *A. pegreffii* in mackerel collected in the Norwegian Sea strongly indicate that the fish became infected as juveniles in areas much further south such as the southern Bay of Biscay. Thus, *A. pegreffii* may prove a useful supplementary marker to further explore the northerly migration routes, especially of the southern and western spawning components of the Atlantic mackerel stock (see Levsen et al., 2017).

Additionally, small numbers of another *Anisakis* sibling species, *A. physeteris*, were found in a few specimens of Atlantic mackerel, silver scabbard fish, European hake and monkfish caught in the Mediterranean Sea. However, these records were very rare, and no worms were found in the flesh of the hosts. Therefore, *A. physeteris* is unlikely to pose any significant risk to human health in European waters. The same applies also for *A. nascettii* (1 larva) and *A. ziphidarum* (1 larva) which were recorded in the visceral cavity, along with several *A. pegreffii* and *A. simplex* (*s. s.*), in a single scabbard fish from the Alboran Sea.

Especially within the present Mediterranean sampling areas there seem to exist geographical "hotspots" of *A. pegreffii* infections in some fish species, which appear to relate more closely to oceanographic characteristics of the actual localities rather than specific fish host traits. For example, anchovy caught close to the Italian coast of the Central and South Adriatic Sea showed comprehensively higher *A. pegreffii* prevalence and abundance, both in the viscera and the flesh, than anchovy from all other sampling localities in the Mediterranean Sea. Interestingly, however, the anchovy from the Adriatic infection "hotspot" area were smaller (mean TL < 130 mm) than their congeners from the other Mediterranean fishing grounds, which were larger (mean TL > 130 mm) but showed considerably lower *A. pegreffii* infection levels. This may indicate that specific oceanographic or ecological factors at the actual fishing area have greater effect on the *A. pegreffii* infection level than specific fish host characteristics such as body size (see Cipriani et al., 2017a).

Considering that *E. encrasicolus* has been reported as the main source of human cases of anisakiasis in certain regions of Italy (Moschella et al., 2004; Mattiucci et al., 2011, 2013) and Spain (Daschner et al., 2000), mostly after the consumption of raw homemade marinated anchovies, the present epidemiological data of *A. pegreffii* in the flesh of anchovy represent important data to evaluate the consumer exposure risk associated with the parasite. Thus, there could be a relation between the high *A. pegreffii* infection levels of anchovy from the Adriatic Sea and some clinical data on cases of anisakiasis in Italy. Interestingly, most cases of human anisakiasis in Italy were actually recorded in some regions which are in comparatively close vicinity to the coast of the Central Adriatic Sea (S. Mattiucci, pers. observation).

3.2.4. Anisakis spp. exposure risk profile

In Fig. 10, we introduce a graphical *Anisakis* spp. exposure risk profile including seven (7) fish species which we consider at highest risk to act as source of anisakiasis in Europe. All species covered in the profile are more or less frequently consumed in a raw or only lightly processed state, without prior freezing, or may be served under-cooked, i.e. if the heating treatment is inadequate, not reaching 60 °C throughout the product. The present risk categories (from *no risk* to *very high*) are based on prevalence data of *Anisakis* spp. larvae in the flesh of the actual fish species. Additional information with regards to particular fishing locality or processing mode of products (e.g. whole or filleted; trimmed or untrimmed fillets) is provided whenever appropriate. It is important to note, however, that for some of the species included in the profile, e.g. Atl. mackerel, herring and cod, trimming of the fillets by removing the ventralmost parts of the belly flaps (see Fig. 3), would reduce the probability of larval presence in the final (fresh) product by more than 90%.

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