Ascaridoid nematode infection in haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) in Northeast Atlantic waters

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Abstract

Haddock and whiting are two species of the family Gadidae that are exploited by fisheries mainly in European waters, the former being one of the most important seafood resources in Scotland (UK). The present study aimed to quantify prevalence, abundance and intensity of infection of the zoonotic parasite *Anisakis* spp. and other ascaridoid nematodes in these fish as part of a study on the risk to consumers. Fish were sourced from research trawling surveys during 2013-2015, aiming to cover a range of size-classes and different times of year. Samples were obtained from the North Sea, west coast of Scotland and the Barents Sea. Fish were stored frozen prior to separation of viscera and fillets and the use of pressing and UV visualisation to locate, identify and count *Anisakis* spp. and other ascaridoids in fillets (divided into 8 parts), liver and other viscera. A subsample of *Anisakis* spp. collected from haddock were identified genetically by means of two diagnostic markers (mtDNA *cox2* and EF1 α -1 nuclear DNA), and all were identified as *A. simplex* (s. s.).

Both fish species showed moderate to high infection levels with *Anisakis* spp. (likely *A. simplex* (s. s.)) in the visceral organs and cavity, and rather low infection in the musculature. Those *A. simplex* (s. s.) larvae present in the musculature were mostly found in the anterior ventral area. In both fish species, the infection rate increased with fish length and in haddock a negative relationship was observed between *Anisakis* spp. numbers in muscle and body condition.

From our sampling of *Anisakis* spp. in fish muscle, in Scotland, whiting presents a greater human health risk than haddock in Scotland. The risk arising from consumption of haddock from the Barents Sea (with 72% prevalence of *Anisakis*) is markedly higher, although in all cases the risk of infection in humans can be minimised if fish are frozen or adequately cooked prior to consumption. We present previously unpublished information on reported cases of anisakiasis in the United Kingdom during 2000-2013, which included 4 cases from Scotland among 22 overall. While these findings suggest that anisakiasis is relatively rare in the UK, underreporting is likely and further investigation of the incidence of anisakiasis is needed.

Keywords

Anisakis simplex (s. s.), zoonotic parasite, haddock, whiting, infection levels

48 Introduction

49 Haddock (*Melanogrammus aeglefinus* L.) and whiting (*Merlangius merlangus* L.) are
50 commercially important gadid species, widely distributed in the Northeast Atlantic. Haddock
51 is the most important whitefish species for the Scottish fishing industry as well as being “the
52 traditional whitefish of choice in Scotland” (Seafood Scotland, 2012) while whiting was not
53 widely valued until the late 20th century, coinciding with low abundance of the heavily fished
54 cod stocks. Most stocks of these two species are currently heavily exploited. During the past
55 decade, annual catches of 30,000–35,000t of haddock and 8,000–12,000t of whiting (valued
56 at £35–50 million and £8–12 million, respectively) were reported for UK waters (Marine
57 Management Organization, 2015).

58 Haddock is especially abundant in the North Sea and adjacent waters but its range extends
59 to Iceland, Barents Sea, Iberian Peninsula, Azores, Greenland, Canada and northeastern
60 USA. It is a medium-large demersal (bottom living) gadid, typically found at depths of 40–
61 300 m, with juveniles preferring shallower, inshore waters. Adult fish can attain a maximum
62 size of 110 cm L and 16 kg W. Haddock can live for 14 years, although ages >9 years are
63 rarely reported (Cohen et al., 1990), and mature at 4–5 years (30 cm L). Spawning occurs
64 annually, over gravel substrates at 6–8 °C, with the fertilised eggs becoming buoyant and
65 developing as they drift in the water column (Bigelow and Schroeder, 1953). Haddock feeds
66 primarily on small benthic invertebrates (worms, echinoderms, crustaceans and molluscs),
67 although very large individuals may consume other small fish (e.g. sand eels and capelin)
68 (Cohen et al., 1990; Jiang and Jørgensen, 1996; Waterman, 2001).

69 Whiting is most abundant in shelf waters around the UK but its distribution extends to the
70 Norwegian Atlantic coast, Baltic Sea, Iceland, Iberian Peninsula, northern Mediterranean
71 and Black Sea. A medium-sized benthopelagic gadid, it is most commonly found in
72 shallower waters, at depths of 20–100 m. Immature fish are found very close to the shore
73 (5–30 m) while larger juveniles and adults are usually found in deeper waters, over muddy
74 and sandy bottoms (Cooper, 1983; Gerritsen et al., 2003). Adult fish can attain a maximum
75 size of 70 cm L and 3 kg W (Cohen et al., 1990). Whiting mature at 2 years (25 cm L)
76 (Cooper, 1983) and spawning occurs annually, over soft (mud-sand) substrates (Wheeler,
77 1969). The spawning period varies with latitude, typically occurring during April to September
78 in UK waters (Hislop et al., 1991). Whiting is an active predator, feeding on many small fish
79 and invertebrate species, including juveniles of commercially important fish species as well
80 as shrimps, crabs, molluscs, polychaetes and cephalopods). Larger whiting feed mainly on
81 fish (Cohen et al., 1990; Hislop et al., 1991).

82 Anisakid nematodes of the genera *Anisakis*, *Pseudoterranova* and *Contracaecum* are
83 roundworms that parasitize marine mammals and fish-eating birds as final hosts. They are
84 characterised by complex life cycles, with their larval stages commonly found in many small
85 crustacean, cephalopod and fish species which serve as intermediate or paratenic
86 (transport) hosts in the marine ecosystem (Mattiucci and Nascetti, 2008; Klimpel and Palm,
87 2011). The third-stage (L3) larvae of these anisakids are commonly found in gadid fish in
88 North Atlantic and Mediterranean waters (Rae, 1963; 1972; Wootten and Waddell, 1977;
89 Wootten, 1978; Smith, 1984; Piccolo et al., 1999; Hemmingsen et al., 2000; Karl et al., 2002;
90 Mattiucci and Nascetti, 2008; Skov et al., 2009; Mladineo and Poljak, 2014; Horbowy et al.,
91 2016; Cipriani et al., 2017a, this issue; Gay et al. 2017, this issue).

These anisakids are known to be of zoonotic importance. While most larval anisakids appear to remain in the fish host's visceral organs, some may migrate into the flesh, and may thus infect humans who consume raw or lightly cooked fillets (or small whole fish). Infection with live zoonotic anisakids exposes humans to the risk of developing the disease called anisakidosis (also known as anisakiasis when caused by *Anisakis* spp.) (Chai et al., 2005; Mattiucci and Nascetti, 2008; Audicana and Kennedy, 2008; EFSA-BIOHAZ, 2010; Hochberg and Hamer, 2010; Mattiucci et al., 2011, 2013, 2017a, b).

Anisakiasis is considered to be an emerging disease of worldwide concern (McCarthy and Moore, 2000; EFSA-BIOHAZ, 2010; Mattiucci and D'Amelio, 2014). Historically, Japan had the highest reported incidence of anisakiasis, up to around 3,000 cases per year (Audicana et al., 2002; Yorimitsu et al., 2013), as compared with approximately 500 cases in Europe and 70 cases in the United States (Arizono et al., 2012). However, it was recently estimated that around 8,000 anisakiasis cases per year in Spain could arise from consumption of raw and marinated anchovy meals, suggesting that anisakiasis is routinely underreported and underdiagnosed (Bao et al., 2017a). *Anisakis* spp. can also cause allergic responses in humans (Audicana and Kennedy, 2008; EFSA-BIOHAZ, 2010; Nieuwenhuizen and Lopata, 2014; Nieuwenhuizen, 2016; Mattiucci et al., 2013, 2017c).

While infected fish tissue may be made safe for consumption by freezing or cooking (EFSA-BIOHAZ, 2010), serious allergic reactions to *Anisakis* spp. antigens have been reported even after these processes have been applied, (Audicana et al., 2002; Audicana and Kennedy, 2008). The potential human health hazards associated with anisakid infestation, combined with the aesthetically unappealing appearance of large numbers of nematodes in fish fillets, represent a serious concern for consumers, food safety authorities and the fishing and food industries (D'amico et al., 2014; Larena-Reino et al., 2015; Bao et al., this issue).

In the United Kingdom, few reports of anisakiasis have been published. Anisakiasis first seems to have attracted attention in 1985, when at least two cases were described (Lewis and Shore 1985; Lucas et al. 1985). Kark and McAlpine (1994) mention that anisakiasis cases have been recorded in the UK but we were unable to find any recent published data.

Anisakids are thought to be widely, albeit patchily, distributed in host fish populations in most geographic areas. To date, nine species of *Anisakis* have been described, including three sibling species of the *A. simplex* complex (*A. simplex* (s. s.), *A. pegreffii* and *A. berlandi*), of which *A. simplex* (s.s.) and *A. pegreffii* are known to be associated with anisakiasis. *Anisakis simplex* (s. s.) is the predominant species in the Northeast Atlantic, North Sea and adjacent waters (Mattiucci and Nascetti, 2008; Kuhn et al., 2011; Mattiucci et al., 2014, 2017a).

There have been relatively few previous studies on the occurrence of anisakids in haddock and whiting in the Northeast Atlantic, most of which examined only a small number of specimens (Elarifi, 1982; Piccolo et al., 1999; Karl et al., 2002; Skov et al., 2009). Smith (1984) reported differing average abundances of *Anisakis simplex* (s. l.) in whiting over two years off northern Scotland (averages of 18 and 114 worms in 1978 and 1979 respectively). Klimpel and Rückert (2005) reported *Hysterothylacium aduncum* in samples of haddock and whiting from the North Sea. In a wider study, Wootten (1978) recorded the occurrence of *Anisakis* spp., *Contracaecum* spp. and *Hysterothylacium* spp. in various fish species from the North Sea, including whiting and haddock. Prevalences and abundances of all nematode genera were higher in larger fish, reaching 100% infection in larger haddock (although mean

worm burdens never reached double figures). While *Anisakis* spp. and *Hysterothylacium* spp. were found in all samples of haddock and whiting, *Contracaecum* spp. was recorded only in whiting from the northern North Sea.

The aim of the present study was to analyse available data on the distribution and infection levels of anisakids in samples of haddock and whiting from EU waters. Our samples came from waters off Scotland (UK) and (in the case of haddock) the Barents Sea, important fishing areas for these species. We have also investigated biological and environmental factors influencing the *Anisakis* spp. presence and abundance in the fish sampled. Since no recent published data are available on the incidence of anisakiasis in Scotland or the rest of the UK, we also summarise available recent information on this zoonosis. Results will help inform fishery managers and consumers and facilitate assessment of associated health risks.

Methods

Data collection, screening, exploration and identification

Data sets on nematode parasites in haddock and whiting in the Northeast Atlantic were collected as part of the EU FP7 PARASITE project (GA no. 312068), which focused on the major commercial seafood species and their most important parasites. Samples of haddock and whiting were collected from a commercial freeze trawler in the Barents Sea (ICES Area I; haddock only, N=150) and from research vessel (RV) surveys of the North Sea (Area IVa, N=115) and West of Scotland (Area VIa, N=176). All samples were collected in 2013 and 2014. Captured fish were immediately frozen on-board and later transferred to the laboratory, where they were later thawed, measured and examined for nematodes.

The thawed fish were first examined externally and measured, and then filleted. The viscera and left and right (side) fillets were separated and placed in large polythene (fish) bags for pressing. Each pressed fillet was sub-divided into four approximately equally sized parts (anterior dorsal, posterior dorsal, anterior ventral (belly flap), posterior ventral), by marking the outside of the bag with horizontal and a vertical lines, bisecting the fillet, with a marker pen. Worms were then counted separately in each fillet sub-division. The digestive system, liver and gonads were also separated prior to pressing, and separate counts of worms were obtained for the different visceral components.

The UV/press-method (Karl and Leinemann, 1993) is increasingly used for the systematic detection of nematode larvae in fish flesh, 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, this issue). The method makes use of the fluorescence of frozen anisakid larvae (Pippy, 1970) and is based on visual inspection of flattened/ or pressed and subsequently deep-frozen fish fillets or viscera under UV-light. A modification of this technique was used, as follows: samples were stored frozen prior to analysis; after thawing, a hydraulic press was used to flatten the fillets and visceral organs of dissected fish before examination under UV light; the viscera were also visually inspected for ascaridoids by the candling technique (i.e. shining a bright light through the viscera placed on a table, in a darkened room). The study was focussed on the zoonotic anisakids, mainly *Anisakis* spp., but also recorded other anisakids such as *Pseudoterranova* spp. and *Contracaecum* spp., and the raphidascarid *Hysterothylacium* spp. All data sets were screened for errors and any

apparent errors checked with sources and corrected if appropriate. In addition to data on individual fish sampled, we generated aggregated data at relevant spatial and temporal scales for the North Atlantic (ICES subdivisions) and season. Finer resolutions were used when possible.

For each fish specimen, the following data were recorded: sex, maturity, total length (TL, mm), total weight (TW, g), liver and gonad weights (g), and total numbers of nematodes of each genus in viscera (gut, liver, gonads) and muscle tissue (fillets, belly flaps). Full data were available for only 100 of the 150 Barents Sea samples. In the remaining 50, nematodes in the viscera were not recorded.

Subsamples of ascaridoids (up to 10 per fish) collected from haddock and whiting were preserved in 4% buffered formalin and/or 70% ethanol and later examined microscopically to confirm identification, referring to published descriptions by Smith and Wootton (1984a, b) and Berland (1991). Some nematodes were cleared in lactophenol to facilitate observation of internal diagnostic features. During processing of the fish, several nematodes were collected from 32 haddock and 18 whiting, Ethanol-preserved, and then shipped for species identification, based on genetic/molecular markers, to the laboratory at the Section of Parasitology (Sapienza-University) in Rome, Italy. Due to a combination of loss of some material in transport and poor preservation in some samples, DNA extraction was finally limited to 42 *Anisakis* spp. from 13 haddock, of which only 22 *Anisakis* from 8 fish specimens (all collected in February 2013) were successfully identified to species level. For specific identification, a multi-marker nuclear genotyping approach was applied: sequence analysis of the mitochondrial *cox2* (mtDNA *cox2*) and elongation factor EF1 α -1 nuclear DNA gene loci (Mattiucci et al., 2016).

The total DNA was extracted using the CTAB method as described by Mattiucci et al. (2014). Thus, the mitochondrial cytochrome c oxidase subunit II (*cox2*) gene was amplified using the primers 211F (5' -TTTCTAGTTATATAGATTGRTTYAT-3') and 210R (5' -CACCAACTCTTAAATTATC-3') spanning the mtDNA nucleotide position 10,639-11,248, as defined for *Ascaris suum* [GenBank X54253]. The PCR conditions followed those reported by Mattiucci et al. (2014). The sequences obtained were compared with those previously published in GenBank for *A. simplex* (s. s.) (DQ116426), *A. pegreffii* (JQ900761), *A. berlandi* (KC809999), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433) and *A. paggiae* (DQ116434).

The elongation factor (EF1 α -1 nDNA) nuclear gene was amplified using the primers EF-F (5' -TCCTCAAGCGTTGTTATCTGTT-3') and EF-R (5' -AGTTTTGCCACTAGCGGTTCC-3') according to Mattiucci et al. (2016). The PCR conditions followed those described by Mattiucci et al. (2016). The sequences obtained at the EF1 α -1 nDNA gene were compared with those previously obtained from *A. pegreffii* (KT825684) and *A. simplex* (s. s.) (KT825685) (Mattiucci et al., 2016).

Information on the incidence of anisakiasis

Information on the number of anisakiasis cases (patients recorded as seropositive for *Anisakis*) reported in the United Kingdom was provided (in 2014) by staff at the Scottish Parasite Diagnostic and Reference Section at Scottish Microbiology Reference Laboratories,

Glasgow, and covered the period 2000-2013. A similar request was made to staff at the Royal College of General Practitioners Research and Surveillance Centre who indicated that there were no recorded cases of seafood-related parasitism.

Descriptive statistics

Summary statistics were computed for several quantitative descriptors of haddock and whiting biometrics (TL, TW) and nematode parasite infection (prevalence, abundance and intensity (i.e. abundance in infected fish), following Bush et al., 1997). Descriptors of parasite prevalence were derived for muscle, viscera and for muscle and viscera combined.

Given that the statistical distribution of numbers of anisakids present in individual fish tends to be skewed, approximating to a Poisson or negative binomial distribution, statistical comparisons of numbers of worms in different parts of the fish (dorsal vs ventral, anterior vs posterior, left vs right) were carried out by first extracting the subset of fish with *Anisakis* spp. in their muscle, then calculating the difference between the numbers of worms in, say, left and right for each fish and applying a Wilcoxon one sample signed rank test. We used a similar approach to compare abundance of *Anisakis* spp. in liver and other viscera in both species. Descriptive statistics and Wilcoxon tests were completed using Minitab 17 (Minitab Inc).

Statistical models for parasite distribution and abundance.

Anisakis spp. presence and abundance in haddock and whiting was analysed for variation related to length, body condition, sex, area and season. A Generalised Additive Modelling (GAM) framework was used (Zuur et al., 2007, 2010), allowing us model non-linear effects of several explanatory variables. Data exploration, model fitting and model validation followed standard procedures (Zuur et al., 2007, 2010; Zuur and Ieno, 2016) using R and BRODGAR (Highland Statistics Ltd).

Presence was modelled using binomial GAM. For abundance, we first tried Poisson GAM and if abundance proved to be overdispersed we used negative binomial GAM. The explanatory variables considered in the models were: total length and sex of the fish, a condition index, month and sampling area (i.e. ICES subdivision, I, IV or VI). Total weight, maturity and gonad weight were not included in the analysis due to their strong correlation with fish length. Length, condition and month were treated as continuous variables and their effects fitted as smoothers. Note that technically month is a circular variable but since we did not have data for every month (and none in December) we treated it as an ordinary continuous variable. Sex and area were included as categorical variables. GAMs were fitted by backwards selection. Information on sex was available for relatively few of the sampled whiting and was dropped from the whiting models to avoid substantial reductions in sample size. Although sampling took place over two years, sampling intensity was insufficient to consider year and seasonal effects simultaneously in the model and we therefore excluded year as a factor. Thus any seasonal variation suggested by the models should be interpreted with caution as it could be a combination of seasonal and year-to-year variation.

We initially calculated a body condition index using the relative condition factor developed by Le Cren (1951). To test its validity, we investigated area-, month- and sex-related variation in

the length-weight relationships in haddock and whiting, fitting a Gaussian GAM to logged TW data, with log TL, area, month and sex as explanatory variables. A seasonal pattern in the condition index is likely, at least in older fish, in relation to maturity state. Both haddock and whiting spawn mainly in spring and early summer. We finally used residuals from this model as a condition index (i.e. weight, controlled for length and adjusted for area-, month- and sex-related differences).

Presence and abundance of other nematode genera were markedly lower (see results) and no statistical modelling was carried out. Correlations between fish length and numbers of the other genera were assessed using Spearman's rho (In Minitab).

Results

Fish sampled and length-weight relationships

The average sizes of haddock sampled varied markedly between areas, the Barents Sea fish being considerably larger. Female haddock outnumbered males in samples from all three areas. The whiting sample from the North Sea included a high proportion of juveniles which could not be sexed. Due to the opportunistic nature of sampling, seasonal coverage is uneven but both halves of the year were sampled for each species and area (Table 1).

The final GAM for (log) weight in haddock included effects of log length ($P < 0.0001$), sex ($P < 0.0001$), area ($P = 0.0002$) and month ($P = 0.0014$) (deviance explained (DE) = 98.4%, $N = 400$). The log weight - log length relationship was positive but departed from linearity, with the longest fish being somewhat lighter than expected given a linear relationship. Males were lighter than females of the same length and haddock from the North Sea and West coast of Scotland were heavier at length than those from the Barents Sea. Note that because the Barents Sea fish were almost all longer than the Scottish fish, the non-linearity of the length-weight relationship casts doubt over the validity of Le Cren's condition index (a different underlying length-weight relationship could be interpreted as a difference in condition), which is a further justification for using residuals from the weight-length GAM as a condition index.

In whiting, the final model for log weight included only log length ($P < 0.0001$) and month ($P = 0.0023$) (DE = 99.4%, $N = 517$). Again, the log weight-log length relationship departed from linear, although in this case there was a slight increase in slope in larger fish. Note however that sex could not be included in the model due to the high number of fish of undetermined sex. Again, residuals from the model were used as a condition index.

Genetic identification of the larval ascaridoid nematodes

The subsample of *Anisakis* spp. larvae analysed was assigned to the species *A. simplex* (s. s.), the most prevalent species of the genus *Anisakis* in the Northeast Atlantic area (Mattiucci and Nascetti, 2008; Mattiucci et al., 2017a). In particular, sequencing of the mitochondrial *cox2* gene in these specimens showed a 99–100% match to *A. simplex* (s. s) sequences previously deposited in GenBank (Mattiucci et al., 2014).

This result was confirmed also by the partial sequence of the nuclear gene encoding EF1 α -1, according to diagnostic nucleotide positions of that locus (Mattiucci et al., 2016).

Summary statistics on infection and ascaridoid nematode identification

All haddock sampled from the Barents Sea and in which viscera were examined, were infected with *Anisakis* spp. (mean abundance = 50.5). Prevalence in muscle was 72% (mean abundance = 2.0). Prevalence and abundance were lower in Scottish haddock samples and the proportion of worms in the muscle was also lower. On the west coast 50% of fish were infected, with a mean of 3.4 worms per fish, and only 1% of fish had *Anisakis* spp. in their muscle tissue (Table 2a). The statistical distributions of *Anisakis* spp. abundance were right skewed, with medians always lower than the mean values (Table 2a).

The presence and abundance of *Anisakis* spp. in haddock appear to be related to both size and location (Figure 1a) and these effects are difficult to separate due to the low overlap in the size ranges of fish sampled around Scotland and in the Barents Sea. The lower abundance of *Anisakis* spp. in fish from the west coast of Scotland (compared to the east coast) is also apparent. Visualisation of the data for fillets (Figure 1b) highlights the absence of *Anisakis* spp. in fillets of fish <300 mm in length, as well as the between-areas differences in prevalence.

Prevalence of *Anisakis* spp. in whiting was 50% on the west coast and 43% in the North Sea: mean worm abundance was 7.9 and 6.4 respectively. Prevalence in muscle was again substantially lower (23% and 10% respectively) (Table 2a). The maximum number of *Anisakis* spp. recorded in a single fish was 183, for both haddock and whiting (although the individual haddock with 183 *Anisakis* spp. contained no other nematodes while the individual whiting was also found to be infected by 19 *Hysterothylacium* spp. larvae in its viscera).

Of the other genera, *Contracaecum* spp. larvae were recorded only in Barents Sea haddock, with 36% prevalence, mean abundance of 0.6 worms per fish and no larvae in the muscle (Table 2b). *Hysterothylacium* spp. larvae were found in both species in all areas, with the highest prevalence (31%) and abundance (mean 1.8) in the viscera of haddock from the Barents Sea. Finally, adult *Hysterothylacium* spp. were found in the stomachs of several haddock and whiting specimens from Scotland.

Specimens of *Pseudoterranova* spp. larvae were also found in both fish species from all areas, with low prevalence and abundance values. Again, the highest values were recorded in haddock from the Barents Sea (6% prevalence, mean abundance 0.8) and *Pseudoterranova* was recorded only in the viscera, the maximum numbers recorded in an individual fish being 43 in haddock and 12 in whiting (Table 2d).

Spearman's correlation coefficients between worm numbers and fish length were significant and positive in *Anisakis* spp. ($r=0.662$, $P<0.0005$) and *Contracaecum* spp. ($r=0.418$, $P<0.0005$). For *Pseudoterranova* spp. ($r=0.082$, $P=0.106$) and *Hysterothylacium* spp. ($r=0.079$, $P=0.119$), correlations with fish length were weakly positive but non-significant.

Site of infection of *Anisakis* spp. in fillets and viscera

In haddock, *Anisakis* spp. abundance (Table 3) and intensity were consistently higher in ventral and anterior parts of the musculature than in posterior and dorsal parts. There was also a strong lateral bias with higher numbers of worms on the left side. These findings were confirmed by results of 1-sample sign tests applied to the subset of haddock with *Anisakis*

spp. in their muscle (95% confidence bands for the difference values did not encompass zero)

In whiting, there was a consistent pattern of more *Anisakis* spp. in the anterior and ventral parts of the musculature but no consistent left-right bias (see Table 3 for abundance data). These findings were confirmed by results of 1-sample sign tests.

In the viscera of haddock from Scottish waters, the abundance of *Anisakis* spp. in liver tended to be higher than in the remainder of the viscera. Overall, across all three sampling areas, the intensity of *Anisakis* infections was significantly higher in liver than in the remaining viscera (median intensities were 7 and 2 respectively). In viscera of whiting the difference between liver and other viscera was less consistent and there was no significant difference in intensity of infection between the two tissues (medians 3 and 2 respectively).

Explanatory variables associated with anisakid presence and abundance in haddock

The final binomial GAM for presence of *Anisakis* spp. in haddock (viscera+muscle) included only an effect of total length ($P<0.0001$, $DE=23.6\%$, $N=386$). *Anisakis* spp. presence increased linearly with length.

The final negative binomial ($\theta = 0.738$) model of number of *Anisakis* in haddock included effects of total length ($P<0.0001$), area ($P<0.0001$), month ($P=0.0030$), sex ($P=0.0332$) and a marginally non-significant effect of residual weight ($P=0.0706$) ($DE = 41.8\%$, $N = 350$). Numbers were highest in the Barents Sea and lowest on the west coast of Scotland, higher in males than in females and highest in March-May. Numbers increased with length in fish up to around 400 mm TL (Figure 2). The smoother for effect of condition suggested a weak negative effect; while inclusion of this variable improved the model, its effect was not statistically significant.

The final (binomial) GAM for presence of *Anisakis* spp. in haddock muscle included effects of area ($P<0.0001$), month ($P=0.0421$) and residual weight ($P=0.0565$) ($DE = 49.2\%$, $N=400$). Presence was highest in the Barents Sea and lowest on the west coast of Scotland. There was a weak seasonal trend, with February values being higher than those in June-September (but note that Barents Sea samples were almost all from the final quarter of the year). There was a marginally non-significant decrease in infection prevalence with increasing residual weight.

Anisakis spp. numbers in haddock muscle followed a Poisson distribution and were related to area ($P<0.0001$), residual weight ($P=0.0062$) and month ($P=0.0012$) ($DE = 51.1\%$, $N = 400$). Numbers were highest in the Barents Sea and lowest on the west coast of Scotland, higher around February to April, and higher for fish with lower residual weights (Figure 3).

Explanatory variables associated with anisakid presence and abundance in whiting

The final binomial GAM for *Anisakis* presence in whiting included effects of length ($P<0.0001$), area ($P<0.0001$), month ($P=0.0009$) and residual weight ($P=0.0474$) ($DE = 40.5\%$, $N = 517$). Prevalence of *Anisakis* increased linearly with fish length and (weakly) with residual weight, and declined over the course of the year. Prevalence was higher in the North Sea than on the west coast. *Anisakis* numbers in whiting followed a negative binomial

distribution ($\theta = 0.725$) and the final model included effects of length ($P < 0.0001$) and month ($P < 0.0001$) (DE = 68.8%, N = 517). The length effect was positive but non-linear while the seasonal pattern suggested peaks in February and August and lows in April and November (Figure 4).

The final binomial GAM for *Anisakis* presence in whiting muscle included effects of length ($P < 0.0001$), month ($P < 0.0001$), area ($P = 0.0015$) and residual weight ($P = 0.0393$) (DE = 48.9%, N = 517). Prevalence of *Anisakis* increased non-linearly with fish length and decreased (weakly) with residual weight, and declined after August (Figure 5). Prevalence was higher in the North Sea than on the west coast. *Anisakis* numbers in whiting muscle followed a negative binomial distribution ($\theta = 1.184$) and the final model included effects of length, month and area (all $P < 0.0001$) (DE = 74.4%, N = 517). Similar to the presence model, the length effect was positive but non-linear while the seasonal pattern showed a decrease after August and abundance was higher in the North Sea than on the west coast.

Incidence of anisakiasis in the UK

In the UK, the prevalence of anisakiasis in humans appears to be low. Information provided by the Scottish Parasite Diagnostic and Reference Laboratory indicated that 22 cases of anisakiasis were detected between 2000 and 2013, four in Scotland, 15 in London, 2 elsewhere in England and one of unknown origin. These cases were recorded in both men and women, ranging in age from 20 to 59. The maximum number of cases per year was 5 (in both 2001 and 2004).

Discussion

Which ascaridoid nematodes are found in haddock and whiting?

Although the genetic identification of the *Anisakis* spp. larvae was carried out on a small subsample of larvae, all 22 larvae identified on the basis of two diagnostic markers (mtDNA *cox2* and EF1 α -1 nuclear DNA) were identified as *A. simplex* (s. s.). These results are in accordance with the known geographical distribution of this species, being the predominant *Anisakis* species in the Northeast Atlantic, North Sea and adjacent waters (Mattiucci and Nascetti, 2008, Kuhn et al., 2011; Mattiucci et al., 2017a). *Anisakis simplex* (s. s.) has previously been genetically identified in haddock from Danish waters (Skov et al., 2009) and in whiting from different areas of the Northeast Atlantic Ocean (Levsen et al., this issue).

Larvae of three other nematode genera were recorded in both haddock and whiting, namely *Hysterothylacium*, *Contracaecum* and *Pseudoterranova*, all less prevalent and with lower abundance and intensity values than *Anisakis*, although individual fish sometimes carried substantial numbers. Maximal abundances for *Anisakis*, *Hysterothylacium*, *Pseudoterranova* and *Contracaecum* in individual haddock were 183, 62, 43 and 7, respectively. Adult *Hysterothylacium* were found in the stomach of several individual haddock and whiting. It should be noted that some authors prefer the use of artificial digestion to quantify numbers of *Anisakis* in fish, and Llarena-Reino et al. (2013) recommended optimizing the artificial digestion method for routine screening of food products. However, Karl and Leinemann (2005) argued that the UV/press method was more efficient as it detected fragments of larvae that would be eliminated by artificial digestion. Nevertheless, it remains possible that the UV/press method sometimes leads to numbers of larvae being underestimated.

***Anisakis* spp. in host fish in relation to body size, condition and sex**

The relatively small sample sizes, differences in sizes of fish sampled in different locations, and the patchy seasonal coverage across two years of sampling mean that it is difficult to be confident that the statistical partitioning of variance between the different explanatory variables was reliable. However, some patterns emerged, e.g. in relation to fish length, that were consistent with previously published results.

In both haddock and whiting, fish host size (length) was the most important factor determining the presence and number of *Anisakis* spp. larvae in the fish, although the presence and number of worms in haddock muscle was unrelated to size. A positive relationship between body length and total number of *Anisakis* spp. has been widely reported for a range of host fish species, including cod, whiting, herring, shads, hake and anchovy (Kahlil, 1969; Wootten, 1978; Tolonen and Karlsbakk, 2003; Klimpel et al., 2004; Valero, 2006; Levsen and Lunestad, 2010; Mladineo and Poljak, 2014; Bao et al., 2015; Cipriani et al., 2017a, 2017b, this issue).

The increasing presence and abundance of *Anisakis* spp. larvae with increasing length (size) of the host might be expected for several reasons. Larger fish will generally have ingested a greater number of worms over their lifetime. Furthermore, they may have changed their diets over time to include prey with higher *Anisakis* burdens. Many fish which are piscivorous as adults, such as European hake (*Merluccius merluccius*) and whiting, switch from crustaceans to small fish prey as they develop and grow larger (Mahe et al., 2007). It is also likely that individual *Anisakis* larvae can remain in a single host for extended periods of time which is in turn related to the longevity of L3 larvae in the food chain, for example their ability to remain in the L3 stage in a number of piscivorous fish species, through successive predation events (Pozio, 2013; Gay et al., this issue).

The whiting sampled in Scottish waters were on average smaller than the haddock taken from these waters but had relatively high levels of infection with *Anisakis* spp. – indeed the maximum number of *Anisakis* recorded in whiting was the same as the maximum recorded in the much larger Barents Sea haddock. Smith (1983) observed very high prevalence of *Anisakis* spp. in whiting muscle samples from Scottish waters, particularly in waters east of Shetland (70%) and west of the Hebrides (63%). This may relate to the more piscivorous diet of whiting compared to the more generalist diet of haddock, in which benthic invertebrates are more important until a relatively larger size is reached, after which haddock also switch to a piscivorous diet. Thus, whiting could be exposed to more *Anisakis* spp. larvae than is the case for haddock of a similar size.

Differences between male and female fish were generally non-significant or weakly significant in haddock and could not be tested in whiting. An effect of body condition was retained in some of the final models but this effect was strong only in the model for *Anisakis* numbers in haddock muscle, where a generally inverse relationship was seen between worm abundance and fish condition. An inverse relationship was also seen in whiting but it was only just statistically significant. Positive relationships are more frequently observed in fish, since good condition is likely to be associated with higher food intake and hence higher exposure to parasites. For example, Podolska and Horbowy (2003) found that prevalence of *Anisakis* spp. in herring increased with both length and condition factor. Bearing in mind that numbers of *Anisakis* spp. in haddock muscle were rather low, the statistically significant

negative relationship with body condition may have little biological significance. It may simply reflect the fact that poor condition is likely to be associated with a reduced mass and volume of viscera, making migration of worms into the muscle more likely. Resistance to *Anisakis* infection is documented in some fish species (see review by Buchmann, 2012) but in relation to haddock the explanation for the statistically effect of body condition remains unclear.

Spatial and seasonal patterns in infection rate

While the low overlap in body sizes between samples of haddock from Scotland and the Barents Sea makes comparisons of *Anisakis* burdens in these two areas problematic, this is not an issue for comparisons of the west and east coasts of Scotland. In samples of both fish species, *Anisakis* spp. tended to be more prevalent and more abundant in samples from the North Sea than in those from the west coast of Scotland. While caution is needed when interpreting results from relatively small samples taken over a short period of time, east-west differences between *Anisakis* spp. loads in fish caught in Scottish waters have been reported previously. Wootten and Waddell (1977) observed a greater abundance of *Anisakis* spp. in cod and whiting sampled from the Northeastern North Sea compared to those from off Southwest Scotland. Wootten (1978) reported a greater abundance of *Anisakis* spp. in offshore than coastal samples of small gadids from Scottish waters, and suggested that this may be related to differences in abundance of euphausiid intermediate hosts and/or final cetacean hosts in these waters. Thus, local infection levels observed in host fish may be related to the degree of exposure to larval anisakids, and influenced (directly or indirectly) by the proximity of intermediate and final host reservoirs of *Anisakis* spp.

In Scottish waters, harbour porpoise (*Phocoena phocoena*) is the most abundant cetacean, while minke whale (*Balaenoptera acutorostrata*), white-beaked dolphin (*Lagenorhynchus albirostris*) and common dolphin (*Delphinus delphis*) are also abundant (Hammond et al., 2013). Bilska-Zajac et al. (2015) stated that the most frequent definitive hosts of *A. simplex* (s. s.) in the northern Atlantic and Pacific oceans are minke whale, common dolphin, pilot whale (*Globicephala melaena*), white-beaked dolphin, killer whale (*Orcinus orca*) and striped dolphin (*Stenella coeruleoalba*). Hauksson et al. (2013) found 89% prevalence and an average intensity of around 1.4 million worms in stomachs of minke whales off Iceland, noting that most seemed to be *Anisakis* spp. Gibson et al. (1998) reported high prevalence by *Anisakis simplex* (s.l.) in strandings of harbour porpoise (60%), common dolphin (71%) and white-beaked dolphin (5 out of 6 individuals) in England. Herreras et al. (2004) found an average intensity of 230 *Anisakis simplex* in 35 harbour porpoise sampled in Denmark. The fact that the abundance of porpoise and minke whale is higher in the North Sea than on the west coast of Scotland (Hammond et al., 2013) may thus help explain the difference in infection rates in fish.

Beluga (*Delphinapterus leucas*) and white-beaked dolphin are highly abundant in the Barents Sea (Kovaks et al., 2014) although the former species occurs only in areas much further north than where the haddock samples were taken. In addition, minke whale is common in the southern Barents Sea. Najda et al. (2015) recorded small numbers of *Anisakis* spp. in 4 of 13 belugas sampled in Hudson Bay (Canada) and it seems likely that minke whales make a more important contribution to the *Anisakis* life cycle in the Barents Sea. However, more information is needed on prevalence and abundance of *Anisakis* spp. in different species of cetaceans in the study area before any firm conclusions are possible.

The present study provided some evidence of variation in *Anisakis* infections in fish between seasons. However, due to the opportunistic nature of the sampling, seasonal and year-to-year differences cannot easily be separated; we can say that there was some significant variation in the infection levels in sampled fish over the course of the study period. Seasonal variation in infection levels is not unusual. Generally, the levels of infection by *Anisakis* species observed in wild fisheries from a given geographic area may be strongly affected by the whole suite of ecological conditions which influence population size of any of the host organisms that are involved in the parasite life cycle. In addition to biotic factors (in both definitive and intermediate hosts), abiotic environmental parameters, such as water temperature and salinity, also influence the biogeography and infection dynamics of *Anisakis* species (Mattiucci et al., 2017a, Cipriani et al., 2017a).

***Anisakis* presence and distribution in fish muscle**

Prevalence and abundance of *Anisakis* spp. larvae in fish muscle seemed to be lower in haddock than in whiting in samples from Scottish waters (only haddock was sampled in the Barents Sea so the comparison is not possible for this area). This is likely not related to *post-mortem* migration since all fish were caught in research trawl surveys or by freezer trawlers and were frozen soon after capture.

In both haddock and whiting, *Anisakis* spp. were more numerous in the ventral and anterior parts of the musculature as compared to dorsal and posterior parts. Interestingly, in haddock but not whiting there was a clear tendency for *Anisakis* spp. to be more numerous on the left side of the fish. These results presumably relate to the nature of the digestive system, specifically location of the stomach, pyloric caeca and liver within the fish and the physicochemical conditions found in fish tissues. *Anisakis* spp. larvae may migrate into the muscle because immune response of the fish host is weaker in its musculature (Cipriani et al., 2016). It has also been hypothesized that *Anisakis* spp. tend to prefer fish tissues with high lipid content (Strømnes, 2014; Strømnes and Andersen, 2003; 1998), and such preferences could influence the location of *Anisakis* spp. within fish hosts (Bao et al., 2017b and references therein). Indeed, it was evident in haddock that a high proportion of the *Anisakis* spp. present in the viscera was located in the liver and this pattern was less evident in whiting.

Implications for human health

The presence of nematode worms in fish raises both aesthetic and health issues. Both haddock and whiting are regularly consumed by humans, haddock in particular being the staple ingredient of fish and chips in Scotland. While cooking or freezing will normally kill nematodes, the presence of thermostable allergens from *Anisakis* spp. may pose a risk for consumers, especially those previously sensitized (Audicana and Kennedy, 2008). Evidently, the most relevant information concerns *Anisakis* spp. in the fillets - the viscera will not normally be consumed. In Scotland, it appears that whiting poses a higher risk than haddock, with 23% of North Sea whiting having *Anisakis* spp. in the fillets as compared to around 11% of North Sea haddock (versus 9% in west coast whiting and 1% in west coast haddock). However, *Anisakis* spp. were present in 72% of Barents Sea haddock sampled, suggesting a considerably higher risk to consumers. It should be noted that the risk could well increase if fish are kept on ice for a substantial period, as may occur in Scotland, since viable worms could migrate from the viscera, where they are much more prevalent according

to our sampling, into the fillets. However, as previously mentioned, freezing and adequate cooking of fish will minimise the risk to consumers.

To date the only formal quantitative risk assessment of human health risks due to *Anisakis* presence in fish was a study carried out in Spain by Bao et al. (2017), where the consumption of raw and marinated anchovies (which may contain live *Anisakis*) is common.

In the UK the prevalence of anisakiasis in humans appears to be low, for example with only four cases reported in Scotland over 14 years (2000-2013). Haddock from the Barents Sea are likely to be consumed mainly in Norway and Russia. In Norway, the number of anisakiasis cases is thought to be low (Lin et al., 2012; Faeste et al., 2014). However, evidence from Spain suggests that anisakiasis and allergy to *Anisakis* proteins are often not diagnosed and are therefore underreported (Bao et al., 2017a; Levsen et al., this issue; Moneo et al., 2017). Serdiukov (1993) suggests that there is a high prevalence of anisakiasis in some regions of Russia, but that is normally erroneously diagnosed as some other gastrointestinal disease. While underreporting may be an issue in the UK anisakiasis data (C. Alexander, Pers. Comm.) it is likely that the human risk from consumption of haddock and whiting in Scotland is low.

For “parasite sensitive” markets a partial solution to the issue would be to trim off the anterior and ventral parts of the fillets, and perhaps to exclude left size fillets in haddock. Given the high prevalence of *Anisakis* spp. infections in haddock and whiting, and the heat- and pepsin-resistance of several *Anisakis* spp. allergens (Caballero and Moneo, 2004; Carballeda-Sangiao et al., 2016, 2014; Vidaček et al., 2009), further research on this topic is needed.

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Table 1. Sample composition (year, season, sex) and summary statistics (length) by area for haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) (F = Female; M = Male; U = Undetermined sex. TL = Total Length (mm); TW = Total Weight (g); Med = Median, SD = Standard Deviation.

	Year		Season				Sex			TL						TW					
Area	2013	2014	Q1	Q2	Q3	Q4	F	M	U	Mean	SD	Med	Min	Max	N	Mean	SD	Med	Min	Max	N
Haddock																					
Barents Sea	0	*150	0	17	0	133	82	68	0	583.9	37.4	585.0	485	700	150	1886.5	383.1	1880	1140	2820	150
North Sea	90	25	90	0	25	0	75	34	6	365.6	42.5	364.0	282	553	115	537.3	234.4	490	230	1700	111
West coast	110	46	110	16	0	50	82	59	35	311.1	84.3	328.5	132	475	176	369.7	254.3	335	15	1155	175
Whiting																					
North Sea	10	281	10	0	281	0	46	23	222	159.8	98.2	99	64	365	291	76.6	108.0	8	2	470	291
West coast	160	66	126	0	0	100	57	87	82	247.1	68.1	267	104	365	226	154.2	118.4	145	5	475	226

* 50 of the haddock sampled in Q4 were examined only for parasites in the muscle

Table 2 Ascaridoid nematode infection in haddock and whiting: mean presence (P), abundance (A) and intensity (I) in viscera and muscle tissue together and in muscle, with additional descriptive statistics for total abundance (SD = standard deviation, Med = median, Max = maximum, Min = minimum, N = sample size; note that sample sizes for intensity (NI) are lower as fish with zero presence are excluded) and that in some Barents Sea fish only muscle was sampled.

(a) *Anisakis* spp.

	Viscera and muscle									Muscle				
Species & Area	P	A	SD	Med	Min	Max	N	I	NI	P	A	N	I	NI
<i>Haddock</i>														
Barents Sea	1.00	50.5	38.9	41	3	183	100	50.5	100	0.72	2.0	150	2.7	109
North Sea	0.84	14.5	20.9	6	0	121	111	17.3	93	0.11	0.3	115	2.5	11
West coast	0.50	3.4	7.0	1	0	47	175	6.7	88	0.01	0.0	176	1	1
<i>Whiting</i>														
North Sea	0.43	7.9	20.5	0	0	183	291	18.5	124	0.23	1.2	291	5.1	66
West coast	0.50	6.4	13.4	1	0	110	226	12.7	114	0.09	0.1	226	1.4	20

(b) *Contracaecum* spp.

	Viscera and muscle									Muscle				
Species & Area	P	A	SD	Med	Min	Max	N	I	NI	P	A	N	I	NI
<i>Haddock</i>														
Barents Sea	0.36	0.63	1.2	0	0	7	100	1.75	36	0	0	150		0
North Sea	0	0					111		0	0	0	111		0
West coast	0	0					175		0	0	0	175		0
<i>Whiting</i>														
North Sea	0	0					291		0	0	0	291		0
West coast	0	0					226		0	0	0	226		0

(c) *Hysterothylacium* spp.

Species & Area	Viscera and muscle									Muscle				
	P	A	SD	Med	Min	Max	N	I	NI	P	A	N	I	NI
<i>Haddock</i>														
Barents Sea	0.31	1.8	7.6	0	0	62	100	5.9	31	0	0	100		0
North Sea	0.22	0.9	2.1	0	0	13	111	4.0	24	0	0	111		0
West coast	0.13	0.3	1.0	0	0	5	175	2.4	22	0	0	176		0
<i>Whiting</i>														
North Sea	0.20	1.2	4.1	0	0	37	291	6.1	58	0	0	291		0
West coast	0.12	0.3	1.0	0	0	6	226	2.5	27	0	0	226		0

(d) *Pseudoterranova* spp.

Species & Area	Viscera and muscle									Muscle				
	P	A	SD	Med	Min	Max	N	I	NI	P	A	N	I	NI
<i>Haddock</i>														
Barents Sea	0.06	0.8	5.1	0	0	43	100	13.0	6	0	0	100		0
North Sea	0.01	0.0	0.1	0	0	1	111	1.0	1	0	0	111		0
West coast	0.03	0.1	0.3	0	0	2	175	1.8	6	0	0	176		0
<i>Whiting</i>														
North Sea	0.01	0.1	1.0	0	0	12	291	8.3	3	0	0	291		0
West coast	0.03	0.1	0.4	0	0	5	226	2.2	6	0	0	226		0

Table 3. Location of *Anisakis* spp. infections in muscle tissue and viscera of haddock and whiting: mean numbers in left (L), right (R), anterior (A), posterior (P), dorsal (D) and ventral (V) portions of the musculature, along with overall ratios, based on pooled data for all fish for L/R, A/P and D/V; mean numbers in liver (LI), other viscera (OV) and their ratio, again based on pooled data. Sample sizes given are the number of fish examined (N1), the number with *Anisakis* in their muscle tissue (N2) and the number if *Anisakis* in their viscera. The figures tabulated refer to all fish sampled whereas statistical tests in the text refer to the subsets of fish with *Anisakis* in their muscle or viscera (i.e. intensity data).

Area	L	R	L/R	A	P	A/P	D	V	D/V	LI	OV	LI/OV	N1	N2	N3
<i>Haddock</i>															
Barents Sea	1.40	0.56	2.40	1.77	0.19	9.50	0.21	1.75	0.12	24.55	24.23	1.01	150	109	100
North Sea	0.17	0.12	1.35	0.22	0.07	3.13	0.11	0.17	0.65	11.85	2.41	4.93	115	11	90
West coast	0.00	0.01		0.01	0.00		0.00	0.01		2.71	0.67	4.05	176	1	87
<i>Whiting</i>															
North Sea	0.53	0.63	0.84	0.78	0.38	2.03	0.15	1.01	0.15	2.85	3.87	0.76	291	66	113
West coast	0.07	0.05	1.45	0.11	0.01	8.0	0.02	0.10	0.17	3.77	2.49	1.51	226	20	112

Figure 1. Numbers of *Anisakis* spp larvae in haddock as a function of fish total length for: (a) viscera and muscle, (b) muscle only. Counts were log transformed ($\log(N+1)$).

Figure 2. GAM for number of *Anisakis* spp. larvae in haddock. Smoothers for partial effects of (a) total length (TL) and month.

Figure 3. GAM for number of *Anisakis* spp. larvae in haddock muscle Smoothers for partial effects of (a) residual weight and (b) month.

Figure 4. GAM for number of *Anisakis* spp. larvae in whiting. Smoothers for partial effects of (a) total length (TL) and month.

Figure 5. GAM for presence of *Anisakis* spp. larvae in whiting muscle Smothers for partial effects of (a) total length, (b) month and (c) residual weight.