Post-mortem tissue migration of *Anisakis simplex* (s.s.) larvae (Nematoda: Anisakidae) in three commercially harvested fish species from the Northeast Atlantic: The role of storage time and temperature

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ABSTRACT

Anisakis simplex sensu stricto is a parasite infecting several commercial fish species in the Northeast (NE) Atlantic, known to be the aetiological agent of the human zoonosis anisakiasis. The present study investigated the response of *A. simplex* (s. s.) third stage larvae (identified to species level by mtDNA *cox*2 sequencing) to the storage time and temperature of Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*) and blue whiting (*Micromesistius poutassou*) from NE Atlantic fishing areas. A total of 300 fish per species were divided in batches of 50 individuals straight after catch. Each batch was stored at different temperature conditions (2 °C, 5 °C, 15 °C) for different time intervals (24h and 48h). A batch of 50 fish of each species was frozen immediately after catch and used as control (time zero). All fish were inspected by the UV-press method. Blue whiting was the most infected fish species while mackerel harboured the highest proportion of *intra-vitam A. simplex* (s. s.) larvae in the musculature. In blue withing there was a significant increase in the proportion of larvae in the muscle with increasing storage temperatures (5 °C < 15 °C) and time (24h < 48h). Herring showed a weak trend of increasing parasite infection in the muscle with increasing temperature/time. In contrast, no significant differences of muscle/viscera larval distribution were observed between batches of mackerel stored at different temperatures for different time intervals. Storage temperature and time seem to play a role in the *post-mortem* motility of *A. simplex* (s. s.) larvae in herring and blue whiting. Keeping the temperature at ≤ 2 °C seems to prevent *post-mortem* larval migration into the flesh during fish storage, handling, and transport. Besides abiotic variables, the differences observed in larval *post-mortem* motility in the different fish species are biologically determined, and attributable to species-specific host-parasite interactions.

1. Introduction

Atlantic herring *Clupea harengus* Linnaeus, 1758, Atlantic mackerel *Scomber scombrus* Linnaeus, 1758, and blue whiting *Micromesistius poutassou* (Risso, 1827) are among the largest and most valuable pelagic fisheries in the Northeast (NE) Atlantic Ocean (ICES, 2021). Norway is one of the main suppliers of these fishes in Europe with annual catches representing around 55%, 20%, and 23% of the total landings in the NE Atlantic Ocean, for Atlantic herring, Atlantic mackerel, and blue whiting, respectively (ICES, 2021). They are highly targeted by the

Norwegian coastal- and oceanic going fishing vessels (Profitability survey of the Norwegian fishing fleet, 2019). After being harvested, fish are generally kept onboard in large, refrigerated seawater tanks at <2 °C, and delivered to the industrial plants for processing within a few days. Thereafter, fish are commercialized fresh, frozen, canned, smoked, salted or dried, with the exception of blue whiting, mostly destined to fishmeal production.

Atlantic herring, Atlantic mackerel, and blue whiting in NE Atlantic Ocean are known to be infected with anisakid parasites of the genus *Anisakis* (Nematoda: Anisakidae) (Chía et al., 2010; Gómez-Mateos

et al., 2016; Levsen, Cipriani, et al., 2018; Levsen, Svanevik, et al., 2018; Mattiucci, 2018a; Mattiucci et al., 2017; Guardone et al., 2019; Roca-Geronés et al., 2020). These parasites exhibit heteroxenous life cycles, involving crustaceans as first intermediate hosts, fish and cephalopods as second intermediate/paratenic hosts, and definitive hosts being mainly cetaceans (Mattiucci & Nascetti, 2006; Mattiucci et al., 2018b). Certain *Anisakis* species are considered among the most important biological hazards in seafood products. In particular, *A. simplex* (s. s.) and *A. pegreffii* are responsible for causing a seafood-borne parasitic zoonosis, anisakiasis, when viable larvae infecting the edible parts of fish or squid are ingested (Arai et al., 2014; Audicana & Kennedy, 2008; Bao, Strachan, et al., 2017; D'Amelio et al., 1999; Fumarola et al., 2009; Guardone et al., 2018; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2017; Mladineo et al., 2016; Sugiyama et al., 2022; Umehara et al., 2007; Van Thiel et al., 1960). Further, the ingestion of these larvae may induce gastric, intestinal and gastro-allergic anisakiasis (Lim et al., 2015; Mattiucci et al., 2011, 2013; Mladineo et al., 2016), and provoke allergic reactions in humans (reviewed in Bao et al., 2019; Daschner et al., 2000). The potential human health hazards associated with the presence of these parasites species in fish, combined with their repellent appearance if present in large numbers, represent a serious concern to the consumers, food safety authorities and fishing industries worldwide (Bao, Strachan, et al., 2017, 2019; D'Amico et al., 2014; Levsen & Karl, 2014).

In fish, most *Anisakis* larvae reside encapsulated in the viscera, while a fraction of these larvae of some specific species (i.e., *A. simplex* (s. s.), *A. pegreffii, A. typica* sp. A and *A. typica* sp. B) may migrate into the muscle (Cipriani et al., 2018, 2022; Karl & Levsen, 2011; Levsen, Svanevik, et al., 2018; Llarena-Reino et al., 2013; Mattiucci et al., 2018a). This migration occurs at some extent when fish are alive (i.e., *intra-vitam* migration) (Cipriani et al., 2014, 2016, 2018, 2022; Karl, 2008; Karl et al., 2002; Quiazon et al., 2011; Smith, 1984) and, under certain conditions, also after fish death (i.e., *post-mortem* migration) (Cipriani et al., 2016; Hauck, 1977; Karl et al., 2002; Roepstorff et al., 1993; Smith, 1983; Smith & Wootten, 1975; Šimat et al., 2015). Temperature and storage time appear to be the most important variables determining the activation and motility of *Anisakis* larvae after fish death (Cipriani et al., 2016; Guan et al., 2021; Karl et al., 2002; Smith & Wootten, 1975). Moreover, studies conducted on herring, anchovies, haddock, saithe and mackerel (Cipriani et al., 2016; Karl et al., 2002; Roepstorff et al., 1993; Smith & Wootten, 1975) suggest that *post-mortem* motility of *Anisakis* larvae varies depending on the fish host species. The data so far available on the motility of *Anisakis* larvae from the viscera into the flesh of herring is controversial. Smith and Wootten (1975) demonstrated experimentally that a large number of *Anisakis* larvae can migrate from the viscera to the flesh of the herring kept on ice at 37 h after catch. Conversely, no migration of *Anisakis simplex* (s. l.) larvae in the flesh of herrings was observed when the fish was exposed to different storage temperatures, ranging from 0 °C to 10° for up to 5 days (Roepstorff et al., 1993). Cipriani et al. (2016) demonstrated that *post-mortem* larval migration from the viscera to flesh of *A. pegreffii* in anchovies (*Engraulis encrasicolus*) caught in the Mediterranean Sea significantly increased with increasing storage temperature (5 °C and 15 °C) and time (24 h, 48 h, and 72 h). Besides this, it has been demonstrated that temperature is an important physical cue for the parasite species to modulate the gene expression levels of some proteins, which play an immunogenic role, and might be implicated in larval migration (Palomba et al., 2020).

A. simplex (s. s.) is the most common anisakid species infecting the visceral organs and the flesh of many commercially important marine fish species in the NE Atlantic (Levsen, Cipriani, et al., 2018). Infections with third-stage larvae of *A. simplex* (s. s.) have been widely reported in Atlantic herring, Atlantic mackerel and blue whiting from the NE Atlantic (Levsen, Cipriani, et al., 2018; Levsen, Svanevik, et al., 2018; Mattiucci, 2018a; Mattiucci et al., 2017; Roca-Geronés et al., 2020). However, there are no clear information regarding potential *post-mortem* migration of *A. simplex* (s. s.) larvae from the viscera to the

flesh in these fishes.

In most commercial fish species, the body musculature represents the only edible part of the fish. Thus, larvae lodging in the muscle tissue of the fish can remain hidden even in processed seafood products and may pose a potential hazard to the consumer. On these premises, the present study aimed to investigate the tissue localization of *A. simplex* (s. s.) larvae in relation to storage time and temperature in Atlantic herring, Atlantic mackerel and blue whiting from NE Atlantic fishing grounds, in order to document and quantify the *intra-vitam* migration and also the possible *post-mortem* migration of the parasite at different experimental conditions, that mimics the conditions at which the fish may be kept during storage, handling, and commercialization.

2. Materials and methods

2.1. Fish sampling and parasitological analysis

A total of 900 fish, 300 per species (Atlantic herring, Atlantic mackerel and blue whiting) were obtained from three fishing grounds of the NE Atlantic Ocean between April and October 2018 (coordinates and dates of catch in Table 1). For each fish species, 300 freshly caught specimens were divided into batches of 50 fish which were stored in separate plastic bags. Each batch of each fish species consisted of roughly equally sized fish, based on gross visual selection, to minimize any effect of host size or age variation. For 0-h control, a batch of 50 individuals per fish species was immediately deep-frozen (−20 °C) post

Table 1

Number (N), coordinates and date of catch, mean values (± standard deviation) of the total length and weight of the fish, and storage conditions (temperatures and time intervals) of the different batches of the 900 fish, 300 per species (Atlantic herring, Atlantic mackerel, and blue whiting), analysed in the study.

Host species	N	Temperature	Time	Fish total length (cm)	Fish total weight (g)
C. harengus Fished June 2018 N 60°41 E 02°35	50	Control	0 h	27.3 ± 0.13 (24.5–30.0)	172 ± 23 (126–225)
	50	2 °C	24h	27.0 ± 0.11 (24.0–29.5)	184 ± 20 (140–232)
	50		48h	27.3 ± 0.10 (25.5–30.0)	197 ± 16 (165–244)
	50	5 °C	24h	27.0 ± 0.14 (25.0–30.0)	177 ± 20 (112–210)
	50		48h	27.3 ± 0.11 (25.5–30.0)	185 ± 20 (152–252)
	50	15 °C	24h	27.3 ± 0.10 (25.0–29.5)	176 ± 19 (144–232)
M. poutassou Fished April 2018 N 57°50 W 09°48	50	Control	0 h	24.1 ± 0.13 (22.0–29.0)	75 ± 11 (58–112)
	50	2 °C	24h	26.6 ± 0.21 (24.0–32.0)	84 ± 21 (58–142)
	50		48h	26.4 ± 0.18 (21.5–32.0)	85 ± 18 (52–138)
	50	5 °C	24h	26.9 ± 0.17 (24.0–32.0)	88 ± 18 (60–134)
	50		48h	26.8 ± 0.17 (24.0–32.0)	93 ± 19 (68–152)
	50	15 °C	24h	27.2 ± 0.23 (24.0–34.5)	93 ± 28 (62–190)
S. scombrus Fished Sept 2018 N 64°13 E 04°26	50	Control	0 h	37.0 ± 0.16 (31.5–40.5)	466 ± 67 (286–628)
	50	2 °C	24h	36.3 ± 0.15 (34.0–40.5)	459 ± 58 (375–643)
	50		48h	36.1 ± 0.16 (33.0–42.0)	472 ± 66 (372–658)
	50	5 °C	24h	36.9 ± 0.13 (34.0–40.0)	473 ± 57 (358–594)
	50		48h	35.8 ± 0.16 (32.0–39.0)	468 ± 69 (330–698)
	50	15 °C	24h	36.9 ± 0.13 (36.5–40.0)	473 ± 58 (360–580)

catch to kill any worms and to avoid any possible migration. The other batches were stored at different temperature conditions ($<2\,°C$, $5\,°C$, $15\,°C$) and time intervals (24h and 48h post catch) (Table 1). Temperatures were continuously monitored using a data logger.

The experimental storage temperatures were chosen according to the following assumptions: $<2\,°C$ represents the suggested storage condition for fish tank storage/transport/handling according to EU rules (Regulation EC No. 853/2004 of the European Parliament); $5\,°C$ represents an upper margin of error for the recommended temperature and the storage condition of refrigerated seafood in retail stores; $15\,°C$ represents an extreme situation that may prevail at artisanal or open-air fish markets. The time conditions were chosen to match the previous experiments reported in literature. The endpoint of the examination (48 h) was chosen by fish degradation observations, considering that the aim of the experiment was to resemble marketable fish conditions.

After thawing, fish were measured (total body length – TL in mm) and weighed (total body weight – TW in g) and sex determined, before being subjected to parasitological examination. At the end of each trial, the fish of each batch were washed, and the excess water of each bag was filtered with subsequent scrutiny of the sieve content (mesh size of 0.5 mm) for nematodes, following Levsen et al. (2005).

The parasitological inspection of each specimen batches was carried out by the same operators. Fish were dissected, and each fillet and viscera were placed separately in individual plastic bags to be inspected by UV-press method (ISO 23036–1). Ascaridoid larvae recovered from each fish were counted and assigned to genus level by light microscopy and following the diagnostic keys proposed by Berland (1961), such as the presence of boring tooth, presence and appearance of ventricle or intestinal caecum, ventricular appendix and terminal mucron, as well as the position of the excretory pore relative to boring tooth and nerve ring. Only *Anisakis* spp. larvae were considered in this study. *Anisakis* specimens were then washed in physiological saline and stored at $-20\,°C$ for further analysis.

2.2. Genetic identification of larval nematodes

A subsample of 150 *Anisakis* spp. larvae, randomly selected from the six batches of each fish species, was identified to species level by mtDNA *cox*2 gene sequencing.

The total DNA was extracted from ≈2 mg of homogenized tissues from each specimen, using the DNeasy® Blood and Tissue Kit (QIAGEN® GmbH, Hilden, Germany). For sequencing the mitochondrial cytochrome C oxidase subunit II (*cox*2) gene, PCR amplification was performed using the primers 211F (5′- TTT TCT AGT TAT ATA GAT TGR TTT YAT-3) and 210R (5′-CAC CAA CTC TTA AAA TTA TC-3) (Nadler & Hudspeth, 2000). Polymerase chain reaction (PCR) was carried out according to the procedures provided by Mattiucci et al. (2014). The sequences were compared with those already obtained for the same gene in our previous works and deposited in GenBank: *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).

2.3. Infection levels and statistical analyses

Differences in host biometric (total length) between batches of each fish species were assessed by one-way ANOVA or Kruskal-Wallis tests, if normality and homogeneity of variance were violated. Tukey's HSD post-hoc tests were used following significant ANOVA's.

Quantitative descriptors of infection levels with *Anisakis* spp. larvae, i. e., prevalence (P, %), mean abundance (A), mean intensity (mI) $\pm$ SD and infection range (min-max), separately for viscera and flesh, were calculated following Bush et al. (1997). To test the homogeneity of *A. simplex* (s. s.) infection levels among all batches, per fish species, the overall (viscera and muscle) prevalence and median intensities of each

batch were compared respectively by chi-square test and Mood's median test. For each fish species, differences in prevalence and mean abundance between the control batch (fish frozen at time 0) and batches of fish stored at different storage temperature and time, were tested by Fisher's exact test and Bootstrap *t*-test using Q-Parasitology 3.0 web (Reiczigel et al., 2019), respectively. To express the changes in larval infection load between the muscle and viscera with increasing storage temperature and -time, the N larvae in muscle/N larvae in viscera-ratio (Nmusc./Nvisc.*100, hereafter Nm-Nv ratio) was calculated, and compared with χ^2 without Yates correction test. χ^2 tests were run to analyse the differences in relative proportions of *Anisakis* spp. larvae in different sites (muscle, viscera) between groups of variables (storage temperature and -time) per fish species. Significance was set at $p < 0.05$. Statistical tests were run in R (R Core Team, 2020).

3. Results

3.1. Identification of Anisakis spp.

According to morphology, a total of N $=$ 17396 *Anisakis* spp. larvae were collected from viscera and flesh of Atlantic herring, Atlantic mackerel, and blue whiting. According to the mtDNA *cox*2 sequences obtained, all 150 *Anisakis* sp. analysed larvae (50 per fish species) were identified as *A. simplex* (s. s.). The mtDNA *cox*2 sequences obtained (524 bp) matched over 99% with mtDNA *cox*2 sequences of *A. simplex* (s. s.) previously deposited in GenBank (highest identity with the sequences MF358545, KC810002 and MT989557, for larvae obtained from Atlantic herring, Atlantic mackerel, and blue whiting respectively).

Three sequences of *A. simplex* (s. s.) per fish species were deposited in GenBank under the following accession numbers: OR568600, OR568601, OR568602 from Atlantic herring, OR568603, OR568604, OR568605 from Atlantic mackerel, and OR568606, OR568606, OR568606 from blue whiting.

3.2. Infection levels

Data on prevalence (P) and mean abundance (A) of infection by *A. simplex* (s. s.) larvae, and their relative proportions in different infection sites in the three fish species examined, according to different temperatures and time intervals are given in Table 2.

The three fish species showed different levels of infection. The most infected fish species was the blue whiting, with a prevalence of 100% and a mean abundance of 40.54 larvae per fish (Table 2), while herring was the less infected (P $=$ 74%; mA $=$ 3.64, Table 2).

All fish species examined at 0h post catch (control batch) harboured larvae in the flesh, thus confirming that larval migration from viscera to flesh occurs *intra-vitam,* at varying rate (Table 2).

3.3. Statistical analyses

Statistical analyses of fish size (TL) showed that herring and blue whiting total length was homogeneous, not differing significantly between the different fish batches (Table 1). Instead for mackerel, according to one-way ANOVA test, the mean fish length differed between the different batches (Table 1). Concerning the homogeneity of *A. simplex* (s. s.) infection levels among all batches, prevalence and median intensity values resulted homogeneous for all fish species (p always >0.05).

3.4. Atlantic herring

Herring showed a weak trend of increasing parasite infection in the muscle with increasing temperature and storage time (Table 2), although neither abundance nor intensity differed significantly between batches stored at $2\,°C$ and $5\,°C$. The Nm-Nv ratio shows a considerable increase with rising temperature and time of storage (Nm/Nv values in

Table 2

Prevalence (P, %), mean abundance (A) and mean intensity (mI and its range) of *Anisakis simplex* (s.s.) in the three fish species for each batch of fish examined, stored in different conditions of temperature and time. Number of total larvae (N_{Tot}) and their relative proportions (%) in different tissues are also given. Nm/Nv represent the number of larvae in muscle/N larvae in viscera ratio (Nmusc./Nvisc.*100). Storage water represents the number of loose larvae collected after sieving the residual liquid from fish box.

Host species			Viscera				Musculature				Storage water	N_{Tot}	Nm/Nv	Total			
			P	A	mI (range)	N_{Tot} (%)	P	A	mI (range)	N_{Tot} (%)	N_{Tot} (%)		% %	P	A	mI (range)	N_{Tot}
C. harengus		Control	72	3.46	4.81 (1-17)	173 (95.1)	14	0.18	1.29 (1–2)	9 (4.9)	0 (0.0)	182	5.20	74	3.64	4.93 (1-18)	182
	2 °C	24h	72	3.42	4.75 (1-23)	171 (94.0)	12	0.18	1.50 (1–3)	9 (4.9)	2 (1.1)	182	5.26	72	3.60	5.00 (1-23)	180
		48h	76	4.16	5.47 (1-20)	208 (96.3)	14	0.14	1.00 (1-1)	7 (3.2)	1 (0.5)	216	3.37	76	4.30	5.66 (1-20)	215
	5 °C	24h	86	3.72	4.33 (1-23)	186 (92.1)	20	0.28	1.40 (1–3)	14 (6.9)	2 (1.0)	202	7.53	90	4.00	4.44 (1-23)	200
		48h	84	4.04	4.81 (1-35)	202 (89.4)	30	0.14	1.20 (1–2)	18 (8.0)	6 (2.6)	226	8.91	88	4.30	5.00 (1-36)	220
	15 °C	24h	76	3.32	4.37 (1-39)	166 (77.2)	32	0.56	1.75 (1–4)	28 (13.0)	21 (9.8)	215	16.87	82	3.88	4.73 (1-43)	194
M. poutassou		Control	100	35.48	35.48 (3–211)	1774 (87.5)	90	5.06	5.62 (1-29)	253 (12.5)	0 (0.0)	1860	14.26	100	40.54	40.54 (4–211)	2027
	2 °C	24h	100	32.98	32.98 (4–184)	1649 (84.5)	92	6.04	6.75 (1-36)	302 (15.5)	1 (0.05)	1952	18.31	100	39.82	39.82 (5–207)	1951
		48h	100	27.10	27.10 (2–142)	1355 (80.7)	96	6.48	6.75 (1-23)	324 (19.3)	0 (0.0)	1679	23.91	100	33.58	33.58 (2–155)	1679
	5 °C	24h	98	21.64	22.08 (2-89)	1082 (83.4)	82	4.30	5.24 (1-21)	215 (16.6)	0 (0.0)	1297	19.87	100	25.94	25.94 (1–110)	1297
		48h	100	25.44	25.44 (1–103)	1272 (79.3)	94	6.66	7.09 (1-45)	333 (20.7)	0 (0.0)	1605	26.18	100	32.10	32.10 (5–148)	1605
	15 °C	24h	100	30.34	30.34 (1–132)	1517 (75.8)	96	9.52	9.92 (1-82)	476 (23.8)	7 (0.4)	2000	31.38	100	39.86	39.86 (3–169)	1993
S. scombrus		Control	100	15.54	15.54 (1-75)	777 (85.4)	80	2.70	3.33 (1-41)	133 (14.6)	0 (0.0)	910	17.12	100	18.20	18.20 (1–216)	910
	2 °C	24h	100	19.24	19.24 (1–105)	962 (87.2)	76	2.82	3.71 (1-25)	141 (12.8)	0 (0.0)	1103	14.66	100	22.06	22.06 (1–130)	1103
		48h	100	17.18	17.18 (1–127)	859 (85.3)	64	2.96	4.62 (1-33)	148 (14.7)	0 (0.0)	1007	17.23	100	20.14	20.14 (2–127)	1007
	5 °C	24h	96	12.70	13.23 (1-66)	635 (89.2)	76	1.54	2.03 (1–5)	77 (10.8)	0 (0.0)	712	12.13	96	14.20	14.83 (1-68)	712
		48h	96	17.38	18.10 (1-57)	869 (87.9)	74	2.40	3.24 (1–9)	120 (11.1)	0 (0.0)	989	13.81	96	19.78	20.60 (1-63)	989
	15 °C	24h	100	17.92	17.92 (1-79)	896 (84.6)	84	3.26	3.88 (1-21)	163 (15.4)	0 (0.0)	1059	18.19	100	21.18	21.18 (1-85)	1059

Table 2, Fig. 1). Herring stored at 15 °C for 24h showed a significant increase of larval abundance in the muscle ($p = 0.007$). Pairwise comparison of the Musculature/Viscera proportions between the control batch and the batches stored at different time intervals showed a general increasing trend (Table 2), with significant differences between the control batch and the fish stored at 15 °C for 24h (χ^2 without Yates correction test, $p = 0.002$) (Table 3).

Finally, increasing number of larvae were recovered from the excess water of the bags of the batches stored at higher temperatures, especially the batch kept at 15 °C for a day (Table 2), indicating that these larvae managed to leave the fish body.

3.5. Blue whiting

In blue withing, there was a significant increase in the proportion of larvae in the fish muscle with increasing storing temperatures and time in all batches, when compared to the control (Tables 2 and 3). The Nm-Nv ratio shows a remarkable increase with rising temperature and time of storage (Nm/Nv values in Table 2, Fig. 1).

When comparing infection levels of the different batches in pairwise comparisons, no statistically significant variations of *A. simplex* (s. s.) prevalence nor mean abundance in the viscera and flesh of the fishes were recorded (in all cases, $p > 0.05$). Pairwise comparison of the relative proportions of larvae counted in fish viscera and musculature between the control batch and those stored at different time intervals showed instead some statistically significant variations (Table 3). A neglectable number of larvae was recovered when sieving the storage liquid in the batches stored at increasing temperature and for longer time (Table 2).

3.6. Atlantic mackerel

In Atlantic mackerel the relative proportions of *A. simplex* (s. s.) larvae in fish viscera and musculature did not follow a linear pattern of increase with storage temperature and time (Table 2).

Pairwise comparison of prevalence and mean abundance in viscera and flesh of the fish between the control batch and the batches stored at 2 °C and 5 °C examined after 24h and 48h showed no significant differences ($p > 0.05$).

The variation of the relative proportions of larvae in fish viscera and musculature with storage temperature and time did not follow a linear increase, even if significant differences were observed between some batches (Table 3). The Nm/Nv ratio did not differ between the control
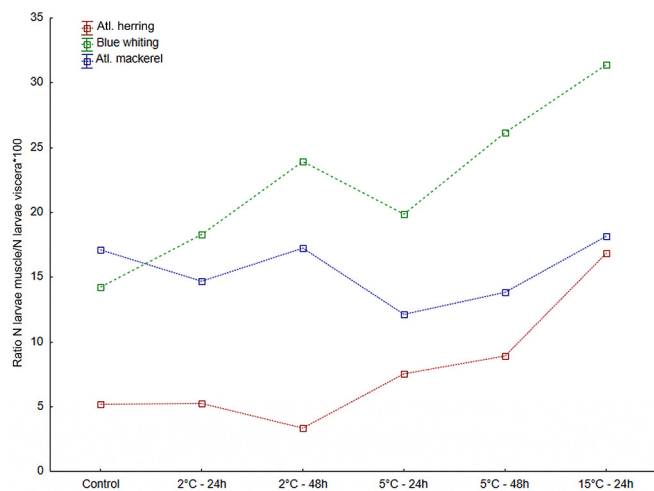
Table 3

Chi-square without Yates correction 2 × 2 contingency table showing the pairwise comparison of the musculature/viscera proportions of *Anisakis simplex* (s.s.) larvae at different intervals of time/temperature. Significance level was set at p > 0.05. "ns" stands for not significant.

Host species			Control	24h
			p	p
C. harengus	**2°C**	24h	ns	–
		48h	ns	ns
	5°C	24h	ns	–
		48h	ns	ns
	15°C	24h	0.002	–
M. poutassou	**2°C**	24h	0.006	–
		48h	0.0001	0.002
	5°C	24h	0.0001	–
		48h	0.0001	0.004
	15°C	24h	0.0001	–
S. scombrus	**2°C**	24h	ns	–
		48h	0.0001	ns
	5°C	24h	0.0237	–
		48h	ns	ns
	15°C	24h	ns	–

and storage trial batches ($p > 0.05$ in all cases) (Fig. 1). No free larvae were recovered from the excess water of the bags of any batches (Table 2).

4. Discussion

Epidemiological data on *Anisakis* spp. larvae in the musculature of the fish provide the basis for analysis and prediction of consumer exposure risk with regards to the presence of anisakid nematodes in commercial fish species. In pelagic fish, the muscle represents the only edible part of the fish, and any larvae present can remain hidden even in processed seafood products, thus representing a potential threat for the consumer. Inappropriate handling and storage conditions of fish can determine the activation and movement of *Anisakis* larvae after fish death (Cipriani et al., 2016; Hauck, 1977; Smith & Wootten, 1975), increasing the rate of *post-mortem* larval migration from the viscera into the muscle tissue, and therefore increase the larval presence in the edible part of the fish. This study investigated the *post-mortem* migration of *A. simplex* (s. s.) in Atlantic herring, Atlantic mackerel and blue whiting at experimental conditions, simulating different conditions for storage, handling, and commercialization of the fish.

Both *intra-vitam* and *post-mortem* larval migration can occur in these fish species. However, while the former is an ecologically driven natural phenomenon, *post-mortem* migration can instead be controlled, mitigated, or completely avoided through proper storage/handling of the fish.

It has been demonstrated that some *Anisakis* species (i.e., *A. simplex* (s. s.), *A. pegreffii, A. typica* sp. A and *A. typica* sp. B, so far) can migrate into the fish muscle *intra-vitam*, and the extent of this movement depends on both host and parasite biology (Cipriani, Sbaraglia, Palomba, et al., 2018; Cipriani et al., 2022, 2022; Hauck, 1977; Levsen, Svanevik, et al., 2018; Roepstorff et al., 1993; Smith, 1983; Smith & Wootten, 1975). The results obtained in this study indicate that *A. simplex* (s. s.) larvae exhibit different infection levels, infection site preferences and motility in the present fish host species obtained from basically the same geographic area. These different patterns of infection are likely determined by fish biology, including feeding habits and host immune responses, as well as host-parasite coevolutionary aspects. In bigger fish species, the extent of larval migration can be also influenced by visceral organ topography (Bao, Strachan, et al., 2017a,b; Smith & Hemmingsen, 2003).

Light microscopy revealed the presence of two nematode types, *Anisakis* type I specimens, and *Hysterothylacium* sp., in all fish host

Fig. 1. Graphic showing the Nm/Nv (N larvae in muscle/N larvae in viscera *100) ratio variations for the three fish species (Atlantic herring, Atlantic mackerel, and blue whiting) according to storage temperature and time.

species examined. *Anisakis* larvae, genetically identified as *A. simplex* (s. s.), occurred both in the viscera and flesh of the examined fish, while *Hysterothylacium* spp., was found only in the viscera. Since *Hysterothylacium* spp. has not been clearly implicated in human infections, and it cannot reach the flesh of the present fish species analysed, it was not considered in this study.

When considering the epidemiology (infection levels per site and the extent of *intra-vitam* larval migration) of *Anisakis* in mackerel, herring and blue whiting studying the control batches, representing the natural pre-catch host-parasite condition (Table 2), the three fish species showed significant differences in infection with *A. simplex* (s. s.). The most infected fish species was blue whiting, while herring was the less infected (Table 2). Differences observed in infection levels may be attributable to the different ecology and biology of the three fish species. Interestingly, the fish species examined showed different levels of infection in the muscle. Mackerel was harbouring the highest proportion of larvae in the muscle (14.6%), referable to *intra-vitam* migration (Table 2), with prevalence P = 80% and mean abundance mA = 2.7, with a single fish hosting 41 *A. simplex* (s. s.) larvae in the muscle. Blue whiting was characterized by the highest parasite prevalence and abundance in the muscle, even if the proportion of larvae in the muscle was lower than the one observed in mackerel (12.5%, see Table 2). Herring had instead low prevalence and abundance of larvae in the muscle (P = 14, mA = 0.18), with only 4.9% of all *A. simplex* (s. s.) larvae seemed to reach the fish flesh. These data were largely in accordance with the literature available for *Anisakis* spp. infections in these fish species from the same geographic area (Levsen, Cipriani, et al., 2018; Levsen, Svanevik, et al., 2018; Levsen & Lunestad, 2010; Mattiucci et al., 2018a; Mattiucci et al., 2017; Roca-Geronés et al., 2020).

The result aimed to estimate *post-mortem* migration by larval *A. simplex* (s. s.) at different temperature and amount of time showed that in fish stored below 2 °C for 24h, no significant variations of infection levels, nor changes in the proportion of larvae invading the fish muscle, were recorded (Tables 2 and 3, Fig. 1). At these conditions, *A. simplex* (s. s.) larvae appear to maintain a latency for at least 24h and do not apparently migrate significantly within the fish host. Further, in herring and mackerel stored below 2 °C, no larval migration was recorded even 48h post catch. However, a significant increase in the proportion of larvae present in fish muscle was detected in blue whiting (p > 0.05, Table 3, Fig. 1) at the same time and temperature conditions. In this fish species, the control batch indicated that 12.5% of *A. simplex* (s. s.) larvae were located in the flesh. At 48h at 2 °C the relative proportion of larvae detected in fish flesh raised to 19,3%, indicating that *post-mortem* migration may occur in blue whiting at this time and temperature conditions. In the same fish species, the relative proportion of larvae in flesh resulted even higher for the batches stored 48h at 5 °C and 24h at 15 °C, raising to 20.7% and 23.8%, respectively (Tables 2 and 3). Also in herring, an increasing number of larvae were detected in the flesh of fish stored at 15 °C for 24h, with the proportion of *A. simplex* (s. s.) larvae raising from a 4.9% in the control batch (representing *intra-vitam* migration) to 13%, thus indicating a remarkable *post-mortem* larval movement at these conditions. Unlike the other fish species, mackerel did not show a clear variation of distribution of *A. simplex* (s. s.) larvae between viscera and flesh when comparing the experimental batches to the control batch. Instead, a peculiar and not linear trend with temperature and time was observed in mackerel, differing from the observations in all the other fish species (Table 2).

The overall trend of variation of larval distribution between the muscle and viscera at varying conditions for the three fish species is illustrated in Fig. 1. The Nm/Nv (N larvae in muscle/N larvae in viscera *100) ratio clearly express the changes in larval infection load with increasing storage temperature and time for herring and blue whiting, while mackerel follow a not linear trend. Considering that the three fish species were exposed to the same standardized conditions of handling, storage temperature and time, and the same inspection method and operators were used throughout, the observed differences in larval *post-*

mortem motility may be attributed to fish biology, or rather to species-specific host-parasite interaction. This hypothesis is supported by the different host reactions to *A. simplex* (s. s.) larvae observed in the three fish species, probably originating from host-parasite co-evolutionary aspects, and strictly linked to fish and parasite interaction. In a recent immunohistochemical study on *A. simplex* (s. s.) in blue whiting, it was reported how the fish apparently oppose a limited reaction to *Anisakis* larvae, with only minor host reactions encountered around the parasite sites of infections (Sayyaf Dezfuli et al., 2021). Thus, the relevant migration of *A. simplex* (s. s.) larvae observed in blue whiting specimens from the viscera to flesh, correlated with storage temperature and time, may also be associated to the mild reaction to *Anisakis* larvae observed in this fish host. In this fish species, the host-induced encapsulation of *A. simplex* (s. s.) larvae would permit the survival of the parasite in a latent condition, with a capsule which could deteriorate rapidly after the fish death, permitting then larval motility.

In herring, the extent of *post-mortem* larval movement was much lower compared to blue whiting. The only significant increase in larval migration into the flesh was observed at 15 °C after 24h of storage. The data so far available on the migration of *Anisakis* larvae from the viscera into the flesh of herring are incongruent, but this could be attributable to the different inspection methods and protocols used. Smith and Wootten (1975) reported that: "… *A large scale migration of Anisakis larvae from the viscera to the flesh of the herring occurred in both our experiments so that almost 20 per cent of the total worm burden was present in the flesh after 37 h*". Hauck, 1977 also reported a significant increase of *Anisakis* s. l. proportions of larvae in the flesh of brined or cold smoked Pacific herring (*Clupea harengus pallasi*) with respect to the 3.5% observed in freshly caught fish. On the contrary, Roepstorff et al. (1993) reported that no migration of *Anisakis* (s. l.) larvae occurred in the flesh of herrings when the fish was kept over a range of different storage temperatures (0° C-10° for up to 5 days).

Apparently, the encapsulation of *A. simplex* (s. s.) larvae in herrings seemed "stronger" than those documented by Sayyaf Dezfuli et al., 2021 in blue whiting (Cipriani, personal obs). The host-induced encapsulation of *A. simplex* (s. s.) larvae in herring resulted thicker, often partially covered by heavily melanised capsules, even if life larvae was present inside the capsule (Cipriani, personal obs). A plausible hypothesis is that host's reactions subsequent to the original larval invasion may have hampered posterior *post-mortem* migration under the time and temperature conditions simulated in the present experiment.

In contrast, a comparatively strong immunological reaction was observed around the infection site of *A. simplex* (s. s.) larvae in Atlantic mackerel, showing many non-viable "*intra-vitam* larvae", which were sometimes even degraded or destroyed. In fact, this fish species seems to oppose a strong reaction towards *A. simplex* (s. s.) larvae, as already observed by Levsen and Berland (2012), with the report of dead and partially disintegrated larvae on the visceral organs and in the fillets. Levsen, Cipriani, et al. (2018) hypothesized that mackerel immunologically reacts strongly, and spends metabolic energy against this apparently harmless parasite.

The results obtained from both control experimental batches, exposed to different storage conditions, show how fish biology can shape the pattern of larval infection in these fish hosts.

Hypothetically, some physical or chemical cues associated with the increase of the temperature may activate *A. simplex* (s. s.) larvae and stimulate their motility *post-mortem* of the fish host. The reason why the tendency of *post-mortem* larval movement is from viscera to flesh (Tables 2 and 3, Fig. 1) is yet to be elucidated. It has been suggested that certain biochemical changes in dead fish, such as accumulation of fatty acid, lactic acid or phosphoric acid, could trigger the *post-mortem* migration of larvae (Hotez et al., 1994; Smith, 1983; Šimat et al., 2015). Recent studies showed that *A. simplex* (s. s.) larvae energy metabolism is based on carbohydrates and fatty acids (Polak et al., 2023; Łopieńska-Biernat et al., 2019). Moreover, *in vitro* studies carried out on *A. pegreffii* under different temperature conditions showed an

association between temperature and gene expression levels of some antigenic proteins released by *Anisakis*, such as peptidases, which could be involved in the host tissue migration of the parasite (Palomba et al., 2020). Furtherly, it has been demonstrated how several proteins having a role in tissue penetration and immunomodulation of the host reaction, carried by microvesicles, are released differentially under temperature-controlled condition in *Anisakis* (Palomba et al., 2023; Guan et al., 2021 demonstrated that the mobility of *Anisakis* spp. larvae in PBS agar varies depending on the temperature conditions. Investigations on the impact of CO_2 and O_2 host tissue content on the larval mobility showed controversial results. While Pascual et al. (2010) found that modified-atmosphere packaging (CO_2, O_2) affected the migration of larvae in fish tissue, Guan et al. 2021observed no meaningful impact of CO_2 and O_2 enriched atmosphere on larval mobility in agar. However, it should be taken into consideration that larval mobility in degrading fish tissues may be different from larval mobility in the artificial texture of PBS agar (Guan et al., 2021).

The present trial showed that both *intra-vitam* migration and *post-mortem* migration of *A. simplex* (s. s.) larvae from the viscera to the flesh of the present fish species does occur. For herring and blue whiting the extent of *post-mortem* larval migration seem to be influenced by storage temperature and time. However, only in blue whiting this larval movement resulted in a statistically significant increase of proportion of larvae invading the muscle, representing the edible part of the fish. In a similar experimental trial investigating *post-mortem* larval migration of *A. pegreffii* in anchovies under similar temperature and time conditions, Authors found that the extent of larval migration from the viscera to the flesh was much higher, with strongest implication for food safety (Cipriani et al., 2016). Aware of the differences of host and parasite species compared to the present study, we can hypothesise that maybe anchovies offer a milder reaction to *A. pegreffii* larvae, as observed by Cipriani et al., 2016, and the smaller size of fish can facilitate the migration from viscera to muscle.

Furthermore, it should be considered that different *Anisakis* species show different capacity of migration into the flesh of other fish hosts, with *A. simplex* (s. s.) showing a higher propensity to invade the flesh of its fish hosts (Cipriani et al., 2014; Quiazon et al., 2011; Ramilo et al., 2023; Suzuki et al., 2010). Indeed, the fish maintained below 2 °C showed none (herrings) to slight (blue whiting) variation in the frequency of *A. simplex* (s. s.) larvae reaching fish musculature.

In the batches kept for 24h at 15 °C it was observed that a small number of *A. simplex* (s. s.) larvae were present on fish surface or free in the boxes. It is not clear if these larvae emerged through the ventral thin muscular tissue of fish, through fish orifices, or from the skin, but their number was negligible. The emergence of some *Anisakis* larvae through natural orifices and from muscle through the skin of anchovies, hakes and blue whiting *post-mortem* has also been previously observed at higher extent (Cipriani et al., 2016, 2018b; Rello et al., 2009).

5. Conclusions

The results obtained in this experimental trial revealed that both *intra-vitam* migration and *post-mortem* migration of *A. simplex* (s. s.) larvae occur from the viscera into the flesh of herring, blue whiting and mackerel. The estimation of *Anisakis* spp. larvae reaching the musculature of the fish is a crucial parameter to assess the health risk associated with the presence of these zoonotic parasites. The *post-mortem* migration of *Anisakis* sp. larvae can be controlled, mitigated, or completely avoided through proper storage and handling of the fish. Results provide useful information on the conditions that can reduce or promote *post-mortem* migration during fish storage, handling, and commercializing, and may contribute to better assess and manage the risk inflicted by the presence of anisakid nematodes in these valuable fish resources.

Keeping the fish at <2 °C throughout the supply chain from capture to consumption, can largely prevent *post-mortem* larval migration from the viscera into the flesh of these fishes. However, a percentage of larvae

occur in the fish already at capture, as a result of the *intra-vitam* migration from the viscera into the flesh. This parameter is related to parasite and host species. Thus, beside strongly suggesting the maintenance of below 2 °C during fish storage/handling or prompt evisceration whenever possible, we also remark the importance of attaining to the general rules of worlds major seafood agencies, recommending the proper heating or freezing of fish before their use in culinary preparations, to prevent any risk of human anisakiasis (EFSA 2010; European Commission, 2011; FDA 2011).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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