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Increasing intensities of *Anisakis simplex* (Rudolphi, 1809 det. Krabbe, 1878) larvae with weight and sea age in returning adult Atlantic salmon, *Salmo salar* L., of coastal waters of Norway

Tor Atle Mo[1] | **Frode Fossøy**[2] | **Trygve T. Poppe**[3]

[1]Norwegian Institute for Nature Research, Oslo, Norway

[2]Norwegian Institute for Nature Research, Trondheim, Norway

[3]Professor emeritus, Oslo, Norway

Correspondence
Tor Atle Mo, Norwegian Institute for Nature Research, Sognsveien 68, NO-0855 Oslo, Norway.
Email: tor.mo@nina.no

Abstract

Ninety wild Atlantic salmon, *Salmo salar* L., (1.5–10.3 kg) were caught in the Namsen Fjord near the mouth of River Namsen, mid-Norway, and examined for the presence and distribution of *Anisakis simplex* (Rudolphi, 1809 det. Krabbe, 1878) larvae by digestion of the viscera and muscles in a pepsin/HCl solution. All salmon were migrating spawners after 1–4 years of feeding in the Atlantic Ocean. All 90 Atlantic salmon had *A. simplex* larvae in the viscera, and all, except two, had *A. simplex* larvae in the musculature. The number of *A. simplex* larvae in each fish varied between 3 and 181, and the total mean number of nematode larvae was 44.5. The intensity of *A. simplex* larvae was positively correlated with increasing weight and sea age of the host. However, the proportion of larvae in the muscle fillets decreased with increasing host weight and sea age. Atlantic salmon females had more *A. simplex* larvae than males. In all the fish examined, 70.2% of the *A. simplex* larvae were found in the viscera and 29.8% in the musculature. The majority (93%) of the larvae in the musculature occurred in the hypaxial sections anterior to the anus. As *A. simplex* larvae commonly occur in the musculature of wild Atlantic salmon, consumption of unfrozen, raw or semi-raw musculature represents a risk for humans developing anisakiasis.

KEYWORDS

Anisakidae, distribution, musculature, RVS, *Salmo salar*, viscera

1 | INTRODUCTION

Marine parasitic nematodes of the genus *Anisakis* Dujardin, 1845 have complex life cycles including hosts at several trophic levels (Mattiucci et al., 2017), and these parasites are commonly found in many animal phyla in the Atlantic Ocean (Shamsi, 2014). Numerous invertebrates, such as crustaceans, and vertebrates, such as fish, act as intermediate or paratenic hosts until eaten by a definitive host such as a whale (Mattiucci et al., 2017). When a larger fish eats an *Anisakis*-infected fish, the larvae can migrate through the wall of the alimentary tract and establish in the tissue of the predator (Mattiucci et al., 2017). Thus, predatory fish may have an accumulated number of *Anisakis* larvae (Mattiucci et al., 2017; Münster et al., 2015).

Anadromous Atlantic salmon, *Salmo salar* L., usually spend one to four years in the Atlantic Ocean before returning to a river to spawn (Hansen & Quinn, 1998). When entering the sea as smolts, the Atlantic salmon start feeding on small prey such as crustaceans and small fish (Haugland et al., 2006). With growth, the prey becomes larger and consists of more and larger fish (see references in Haugland et al., (2006)). As *A. simplex* larvae may be accumulated in piscivorous fish (Klimpel et al., 2004; Strømnes

& Andersen, 2003), an increase in the abundance of *Anisakis* larvae in Atlantic salmon with increasing weight and sea age may be expected.

The presence of *Anisakis* larvae has been reported in Atlantic salmon caught in different geographical areas (Beverley-Burton et al., 1977; Beverley-Burton & Pippy, 1978; Kent et al., 2020; Noguera et al., 2015; Pippy, 1969; Senos et al., 2013; Wootten et al., 2010b). Most of these studies only reported the occurrence of *Anisakis* larvae associated with the viscera in the abdominal cavity of the fish, but some studies included the presence of *Anisakis* larvae also in the musculature (Beverley-Burton & Pippy, 1978; Kent et al., 2020; Noguera et al., 2015) and one in the musculature only (Wootten et al., 2010b).

Consumption of raw Atlantic salmon in sushi dishes and semi-raw as smoked or cured has become increasingly popular in many areas of the world. While *Anisakis* larvae in insufficient processed wild marine fish may cause discomfort and diseases in humans (Audicana et al., 2002; Audicana & Kennedy, 2008; Pascual & González, 2018; Pascual et al., 2018), farmed Atlantic salmon is documented free from *Anisakis* larvae in the musculature (Angot & Brasseur, 1993; Levsen & Maage, 2016; Mo et al., 2014; Wootten et al., 2010a). However, in this context, the occurrence of *Anisakis* larvae in wild Atlantic salmon is of particular interest as the risk difference between farmed and wild salmon is unknown to most residents.

Previously, the presence of *Anisakis* larvae in Atlantic salmon of variable weight, sea age and sex has only been studied once in the northwest Atlantic (Beverley-Burton & Pippy, 1978), but the study included the presence of larvae in the musculature with no comment on the distribution of the larvae in the fillets (Beverley-Burton & Pippy, 1978). Thus, knowledge about the occurrence of *Anisakis* larvae in the fillets with increasing weight in multi-sea-winter (MSW) Atlantic salmon is lacking. Here, we examined 95 Atlantic salmon of variable weight, sea age and sex caught in coastal waters of mid-Norway for the presence and distribution of *Anisakis* larvae in the viscera and the muscular fillets divided into twelve sections.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Ninety-five Atlantic salmon were sampled in bag nets during their migratory spawning run within the period 23 May – 5 July 2013 at two locations in the Namsen Fjord (between 64°36′57.5′′N 10°59′49.2′′E and 64°30′12.6′′N 11°10′10.6′′E), a few km from the outlet of the River Namsen. All fish sampled during this study were captured by two fishermen with official permission to catch fish in bag nets. The fish were killed by a sharp blow to the head, packed individually in plastic bags and put into a freezer (−20°C) within one hour. Later, all fish were transported frozen to the laboratory for further processing.

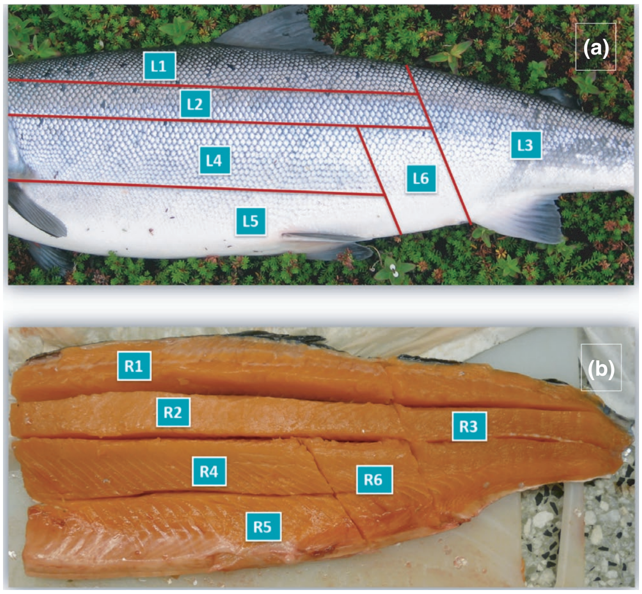

FIGURE 1 Subdivision of Atlantic salmon fillets to study the distribution of *Anisakis simplex* larvae. (a) External division of the right fillet (R1-R6). (b) Internal division of the left fillet (L1-L6). The lower line of sections 2 and upper line of sections 4 and 6 follow the outer lateral line of Atlantic salmon. L1&R1: upper epaxial muscle; L2&R2: lower epaxial muscle; L3&R3: tail muscle, L4&R4: upper belly flap; L5&R5. Lower belly flap; L6&R6: vent part

2.2 | Sample preparation

After thawing, the fish length and weight were recorded. Scales were sampled above the lateral line between the dorsal and adipose fins. The scales were analysed to distinguish escaped farmed Atlantic salmon from wild Atlantic salmon and for determination of the number of years the salmon remained feeding at sea. The fish scales were analysed by official experts in fish scale analysis at the Norwegian Veterinary Institute. A knife opened the fish, the sex noted, and the viscera including the kidney were removed. The viscera including the inside of the alimentary tract were visually examined with the help of two pair of pincers. All visually observed nematodes were put in a separate tube with 96% ethanol labelled with fish number and tissue location. The viscera (stomach, gut, liver, spleen, gonads) were pooled and digested as one section (see below).

The left and right fillets were separated from the head, backbone and tailfin, and the skin was removed. These latter four sections were not examined for the presence of anisakid larvae; that is, only the fillets were further processed. The left (L) and right (R) fillet musculature was cut into six different sections, labelled L1-L6 and R1-R6, respectively (Figure 1), following the subdivision of Karl et al., (2011) with minor adjustments. To quantify the number and distribution of nematode larvae, each section was digested separately.

All 13 sections (viscera + L1-6 + R1-6) were weighted and digested using an enzyme digestion method (Jackson et al., 1981;

Lunestad, 2003). The digestion was carried out in 2-L Erlenmeyer flasks, containing 500 ml of tap water and 4 g of pepsin (Pepsin A 1:10,000 NF granulate from Biotec Marine Biochemicals) per 100 g of fish muscle or viscera. Hydrochloric acid (6 M) was added to the solution to adjust the pH to between 3 and 4. Each solution was continuously stirred and kept at 40°C using thermostat-heated, magnetic stirrer plates, for 6–16 hr, depending on the weight of the viscera or the fillet sections. When the digestion was completed, the hydrolysate was filtered through a sieve, with a mesh size of 1 mm, and flushed carefully with tap water. Trapped parasites were transferred to water in a Petri dish with black bottom (to increase the contrast), counted and preserved in 96% ethanol in a separate tube labelled with fish number and section.

2.3 | Nematode species identification

Nematodes sampled from the viscera by visual inspection and released after digestion of the viscera were separated into "large" and "small" specimens. The "large" specimens differed from the described morphology and morphometry of the genus *Anisakis* (Smith, 1983) and were similar to nematodes identified as *Hysterothylacium aduncum* (Rudolphi, 1802) in Mo et al., (2014). These large nematodes were excluded from further analysis. Only "small" nematodes were released after digestion of the musculature. All the "small" nematodes had similar shape and size, and correspond to the shape and size of larvae of the genus *Anisakis* (Smith, 1983). A subsample of fifty "small" larvae, randomly selected from viscera or musculature, was subjected to molecular diagnostic analysis.

DNA from each nematode specimen was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the protocol. DNA quantity was measured using a NanoDrop spectrophotometer, and the DNA extract was diluted to approximately 15–20 ng/µl.

Approximately 600 bp of the partial CO2 gene was amplified using the reverse primer 210 (5′-CACCAACTCTTAAAATTATC-3′) and a modified version of the forward primer 211; 211B (5′-TTTTCT GGTTATATGGATTGATTTCA-3′), from Nadler and Hudspeth (2000). PCR was performed in a total volume of 10 µl with 4.6 µl dH_2O, 1.0 µl PCR buffer 10× (Qiagen), 0.5 µM of each primer, 1.0 mM dNTP (Thermo Fisher Scientific), 3.0 mM BSA (BioLabs), 0.5 units HotStarTaq DNA polymerase (Qiagen) and 2.0 µl DNA template. PCR amplification was carried out with denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 45°C for 30 s, 72°C for 50 s and a final extension at 72°C for 5 min. Excess of primers and dNTP was removed from the PCR products by treatment with ExoSAP-IT (Applied Biosystems) according to the manufacturer's protocol.

The samples were sequenced from both ends with primer 211B (5′3) and 210 (3′5), using BigDye Terminator v1.1 kit (Applied Biosystems). Reactions consisted of 3.5 µl dH2O, 2.0 µl sequencing buffer 5×, 0.5 µM primer (211B or 210), 1.0 µl BigDye v1.1 and 3 µl 1:10 diluted PCR product in a total reaction volume of 10 µl. Thermal cycling was performed with an initial denaturation at 94°C for 1 min, followed by 25 cycles at 94°C for 15 s, 50°C for 10 s, 60°C for 4 min and a final hold at 4°C. The sequencing products were purified by EtOH precipitation, resuspended in 10 µl HiDi formamide (Applied Biosystems) and sequenced on the 3500×L Genetic Analyser (Applied Biosystems).

2.4 | Data and statistical analysis

Calculation of distributions of nematode larvae by weight, sea age, sex and fillet sections was done in Microsoft Excel, and statistical tests and graphics were fitted in R v3.6.2 (R Core Team, 2020). General linear models (GLM) were fitted to explain the variation in fish weight (using a normal distribution) and total number of parasites (using a Poisson distribution). Generalized mixed models (GLMM) were fitted to explain the distribution of parasites in fillet parts (muscles), including fish identity as a random factor to control for non-independence of fillets within each fish. Generalized mixed models (GLMM) were fitted using the package "lmerTest" (Kuznetsova et al., 2017), and plots were fitted using ggplot2 (Wickham, 2016).

3 | RESULTS

3.1 | Origin of the Atlantic salmon

At necropsy, three of the large Atlantic salmons (>7 kg) were identified as recently escaped farmed fish based on several morphologic characters (body shape, curved fin rays, visceral adhesions). Analysis of their scales confirmed this. No nematodes were found in any of the three fish. Two additional fish (>7 kg) were identified as escaped farmed Atlantic salmon based on scale analysis even if they appeared wild on the several morphological characters. These two fish had 28 and 36 *A. simplex* larvae, respectively, and likely, they had escaped before transfer to marine cages or soon after transfer. All five Atlantic salmon identified as escapees from fish farms were excluded from further analysis.

3.2 | Sex, weight and sea age of the Atlantic salmon

Of the remaining 90 analysed salmons, these comprised of 67 females and 23 males (Table 1). Forty-one (28 females and 13 males) were classified as small salmon or grilse (<3 kg), 25 (22 females and 3 males) were classified as medium weighted salmon (>3 and <7 kg), and 24 (17 females and 7 males) were classified as large salmon (>7 kg) (Tables 1 and 2). Based on scale analyses, 33 salmon (23 females and 10 males) had grown for 1 year in the sea (1SW), 39 salmon (29 females and 10 males) had grown for 2 years (2SW), 14 salmon (11 females and 3 males) had grown for 3 years (3SW), and four salmon (all females) had grown for 4 years in the sea (4SW) (Tables 1 and 3). In the statistical analyses, these four females were included

TABLE 1 Length, weight, sea age, sex and occurrence of *Anisakis simplex* (*A.s.*) in 90 Atlantic salmon

Fish no.	Length (mm)	Weight (kg)	Sea age	Sex	Total *A.s.*	*A.s.* in viscera	*A.s.* in fillets	*A.s.* in left fillet	*A.s.* in right fillet
1	570	1.52	1	♂	18	6	12	5	7
2	580	1.62	1	♂	28	10	18	14	4
3	690	1.63	1	♂	10	3	7	4	3
4	600	1.66	1	♂	32	10	22	14	8
5	620	1.77	1	♀	7	4	3	3	0
6	580	1.78	1	♀	18	2	16	10	6
7	630	1.79	1	♂	47	29	18	13	5
8	590	1.79	1	♂	18	10	8	6	2
9	610	1.87	1	♀	4	3	1	0	1
10	590	1.88	1	♀	4	3	1	0	1
11	615	2.05	1	♂	14	5	9	6	3
12	630	2.08	1	♀	18	10	8	3	5
13	590	2.08	1	♂	12	5	7	5	2
14	665	2.09	1	♀	25	18	7	5	2
15	620	2.10	2	♂	27	10	17	8	9
16	620	2.12	2	♀	10	3	7	6	1
17	660	2.13	1	♀	7	3	4	0	4
18	640	2.17	1	♀	9	3	6	2	4
19	655	2.18	1	♀	94	70	24	14	10
20	625	2.18	1	♀	94	72	22	15	7
21	630	2.19	1	♀	21	9	12	7	5
22	630	2.19	1	♀	32	23	9	4	5
23	730	2.19	2	♀	8	3	5	1	4
24	610	2.21	1	♂	21	14	7	7	0
25	630	2.32	2	♀	17	9	8	4	4
26	645	2.33	1	♂	11	7	4	2	2
27	670	2.38	2	♂	22	18	4	2	2
28	700	2.40	2	♀	38	27	11	4	7
29	645	2.40	1	♀	83	42	41	27	14
30	650	2.43	2	♂	22	9	13	10	3
31	650	2.44	2	♀	20	12	8	6	2
32	650	2.46	1	♀	40	32	8	4	4
33	660	2.51	1	♀	53	46	7	4	3
34	740	2.52	2	♀	53	35	18	9	9
35	620	2.52	1	♀	62	47	15	11	4
36	670	2.72	2	♀	41	32	9	6	3
37	660	2.78	1	♀	18	4	14	4	10
38	670	2.79	1	♀	56	39	17	14	3
39	690	2.83	2	♀	8	7	1	0	1
40	680	2.88	1	♀	53	41	12	8	4
41	660	2.92	1	♀	42	26	16	12	4
42	680	3.03	1	♀	38	32	6	5	1
43	705	3.03	2	♀	38	17	21	13	8
44	670	3.04	1	♀	35	19	16	10	6

(Continues)

TABLE 1 (Continued)

Fish no.	Length (mm)	Weight (kg)	Sea age	Sex	Total *A.s.*	*A.s.* in viscera	*A.s.* in fillets	*A.s.* in left fillet	*A.s.* in right fillet
45	680	3.17	2	♀	63	36	27	7	20
46	690	3.45	2	♀	79	64	15	13	2
47	710	3.50	2	♀	41	30	11	7	4
48	690	3.50	1	♀	43	27	16	9	7
49	765	3.59	2	♀	9	2	7	7	0
50	705	3.61	2	♀	181	107	74	19	55
51	720	3.69	2	♀	36	19	17	13	4
52	730	3.81	2	♀	49	37	12	5	7
53	740	3.82	2	♀	21	9	12	7	5
54	740	3.89	2	♀	25	14	11	3	8
55	730	3.90	2	♀	46	40	6	5	1
56	750	4.03	2	♀	23	14	9	5	4
57	760	4.04	2	♀	82	53	29	13	16
58	745	4.07	2	♀	43	27	16	15	1
59	770	4.14	2	♂	36	25	11	4	7
60	800	5.11	2	♀	36	23	13	4	9
61	815	5.13	2	♂	111	90	21	11	10
62	810	5.14	3	♀	5	1	4	2	2
63	820	5.19	2	♀	33	23	10	8	2
64	830	5.33	3	♀	161	140	21	11	10
65	830	5.94	2	♀	65	55	10	4	6
66	820	6.43	2	♂	80	51	29	21	8
67	830	7.03	2	♂	104	80	24	16	8
68	860	7.05	2	♀	123	107	16	10	6
69	870	7.09	2	♂	36	34	2	2	0
70	880	7.15	3	♀	63	57	6	6	0
71	860	7.22	2	♂	30	28	2	2	0
72	820	7.24	2	♀	111	90	21	8	13
73	935	7.41	3	♂	3	3	0	0	0
74	870	7.53	3	♀	48	28	20	11	9
75	870	7.84	2	♀	18	10	8	2	6
76	875	7.91	2	♀	154	151	3	2	1
77	900	8.09	3	♀	62	55	7	6	1
78	900	8.13	4	♀	28	16	12	5	7
79	860	8.18	2	♂	24	19	5	4	1
80	925	8.18	4	♀	57	21	36	20	16
81	900	8.30	3	♂	16	16	0	0	0
82	920	8.39	3	♂	102	84	18	18	0
83	910	8.41	3	♀	19	9	10	7	3
84	890	8.58	3	♀	83	66	17	9	8
85	930	8.79	3	♀	46	19	27	12	15
86	950	9.43	4	♀	78	57	21	16	5
87	910	9.50	3	♀	56	35	21	16	5
88	920	9.83	3	♀	58	42	16	10	6
89	970	10.11	3	♀	75	53	22	14	8

(Continues)

TABLE 1 (Continued)

Fish no.	Length (mm)	Weight (kg)	Sea age	Sex	Total *A.s.*	*A.s.* in viscera	*A.s.* in fillets	*A.s.* in left fillet	*A.s.* in right fillet
90	985	10.28	4	♀	15	14	1	1	0
Sum					4,005	2,810	1,195	697	498
Mean number *A.s.*					44.5	31.2	13.3	7.7	5.5
Per cent of total no. of *A.s.*					100	70.2	29.8	17.4	12.4

TABLE 2 Occurrence of *Anisakis simplex* (*A.s.*) in three size groups and sex of 90 Atlantic salmon

	<3 kg			3–7 kg			>7 kg		
	All	♀	♂	All	♀	♂	All	♀	♂
No. of salmon	41	28	13	25	22	3	24	17	7
Total mean *A.s.*	29.7	33.4	21.7	55.2	52.4	75.7	58.7	64.4	45.0
Mean *A.s.* in viscera	18.6	22.3	10.5	38.2	35.9	55.3	45.6	48.8	37.7
% *A.s.* in viscera	62.5	66.8	48.2	69.3	68.5	73.1	77.6	75.9	83.8
Mean *A.s.* in fillets	11.1	11.1	11.2	17.0	16.5	20.3	13.1	15.5	7.3
% *A.s.* in fillets	37.5	33.2	51.8	30.7	31.5	26.9	22.4	24.1	16.2

TABLE 3 Occurrence of *Anisakis simplex* (*A.s.*) in three age groups and sex of 90 Atlantic salmon

	1 year in the sea			2 years in the sea			3 and 4 years in the sea		
	All	♀	♂	All	♀	♂	All	♀	♂
No. of salmon	33	23	10	39	29	10	18	15	3
Total mean *A.s.*	32.3	37.2	21.1	50.3	50.7	49.2	54.2	56.9	40.3
Mean *A.s.* in viscera	20.4	25.0	9.9	36.4	36.4	36.4	39.8	40.9	34.3
% *A.s.* in viscera	63.2	67.2	46.9	72.3	71.8	74.0	73.4	71.8	85.1
Mean *A.s.* in fillets	11.9	12.2	11.2	13.9	14.3	12.8	14.4	16.1	6.0
% *A.s.* in fillets	36.8	32.8	53.1	27.7	28.2	26.0	26.6	28.2	14.9

TABLE 4 General linear model (GLM) of number of *Anisakis simplex* larvae in relation to sex, weight and sea age of Atlantic salmon. The number of larvae was fitted using a Poisson distribution

	Estimate	SE	Z	*p*
(Intercept)	3.23	0.04	82.59	<.001
Sex (male)	−0.30	0.04	−7.60	<.001
Weight	0.14	0.01	13.81	<.001
Sea age (2 SW)	0.11	0.05	2.38	.017
Sea age (3 + SW)	−0.39	0.08	−4.99	<.001

in the 3SW category and named 3+SW. Weight was strongly associated with sea age but not by sex (GLM sex: $t = 0.72$, $p = .48$).

3.3 | Nematode species identification

DNA could be extracted from 30 specimens among the subsample of 50 nematode larvae. All 30 larvae were identified as *A. simplex* sensu stricto (Rudolphi, 1809 det. Krabbe, 1878) by Sanger sequencing and identification of the DNA sequence by BLAST (McGinnis &

Madden, 2004). This is in accordance with other studies concluding that *A. simplex* s.s. appears to be the only *Anisakis* species present in the Northeast Atlantic Ocean (Kent et al., 2020; Klapper et al., 2015; Levsen et al., 2018). This is also supported by the observation of Suzuki et al., (2010) that *A. simplex* s.s. has a higher penetration rate into fish muscle tissue than other *Anisakis* species. Thus, it is likely that all the "small" nematodes found in this study were *A. simplex* larvae.

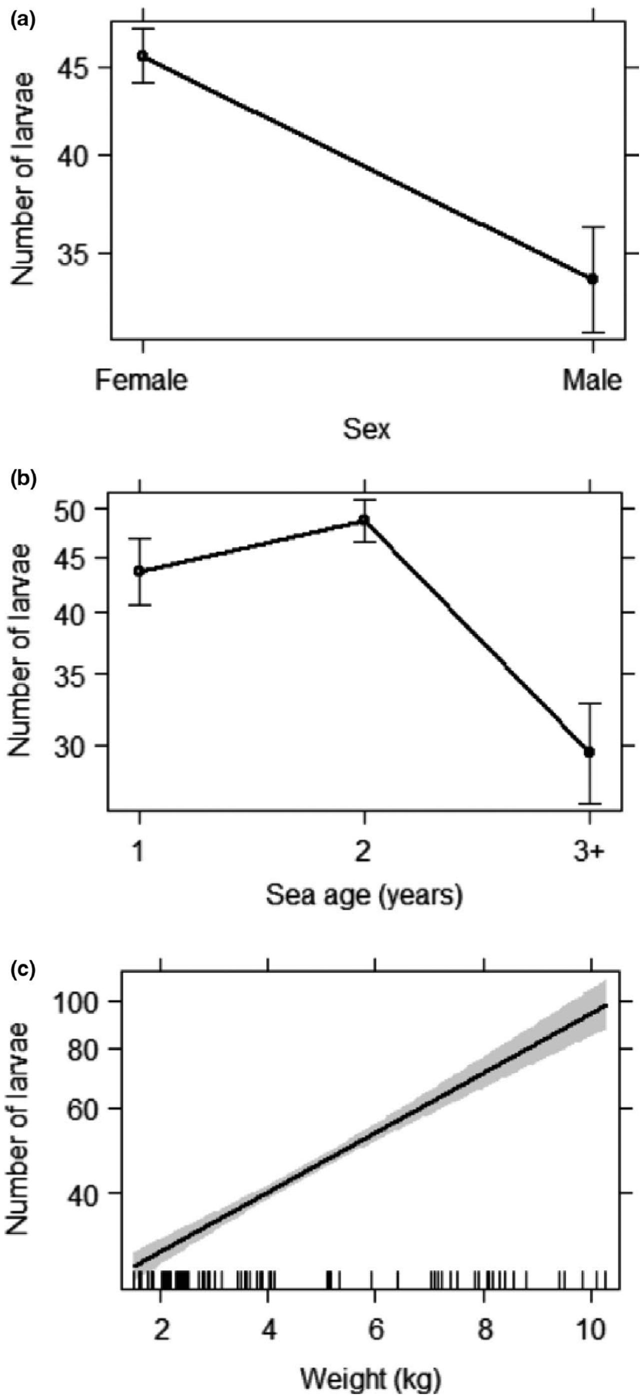

FIGURE 2 Depiction of modelled effects of Atlantic salmon sex (a), sea age (b) and weight (c) on the total number of *Anisakis simplex* larvae as estimated in the general linear model (GLM) in Table 4

3.4 | Abundance of *Anisakis simplex*

Anisakis simplex larvae were found in all 90 Atlantic salmon examined. The number of larvae per fish varied between 3 and 181, in total 4,005 larvae (Table 1). The mean number of larvae for all salmon was 44.5 (Table 1). The total mean number of *A. simplex* larvae in all females (67) and males (23) was 47.5 and 35.8, respectively (not presented). The mean number of nematode larvae in the weight groups and sea age groups is presented in Tables 2 and 3.

A GLM of the total number of larvae in each fish showed association with sex, sea age and weight (Table 4, Figure 2), where females had significantly higher numbers of larvae than males, fish 3 years or older (3+SW) had fewer larvae than younger fish (1SW and 2SW), and larval numbers were positively associated with fish weight. Importantly, these modelled effects show that when controlling for fish weight, there are fewer larvae in large fish per musculature weight unit, although the absolute number of larvae is higher in larger fish (Table 2).

3.5 | Tissue distribution of *Anisakis simplex*

Of the 4,005 *A. simplex* larvae, 2,810 (70.2%) and 1,195 (29.8%) were found in association with the viscera and in the fillets (musculature), respectively (Table 1). Independent of fish weight, the mean number of *A. simplex* larvae in association with the viscera and in the fillets was 31.2 and 13.3, respectively (Table 1). The percentage of larvae found in the fillets in each fish varied between 0% and 88.9% (not presented), which means that some Atlantic salmon specimens had the majority of the *A. simplex* larvae in the fillets and relatively few in association with the viscera. However, there was a positive correlation between the number of *A. simplex* larvae in the viscera and the fillets for both males (Pearsons: $df = 21$, $t = 2.96$, $r = 0.54$, $p = .008$) and females (Pearsons: $df = 65$, $t = 3.57$ $r = 0.40$, $p < .001$) (Figure 3). There was a significant decrease in the proportion of *A. simplex* larvae in the fillets with increasing weight in males (Pearsons: $t = -6.62$,

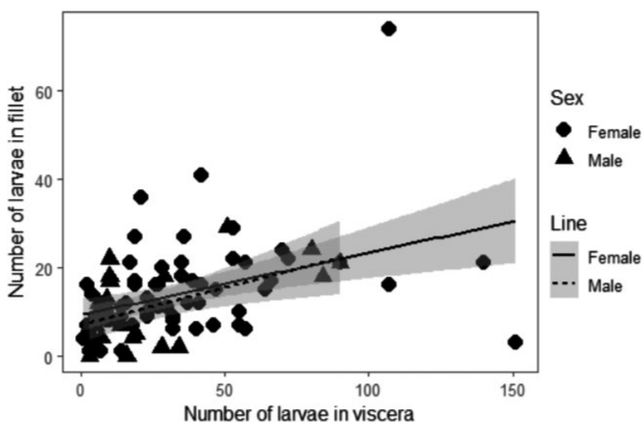

FIGURE 3 Correlation between number of *Anisakis simplex* larvae found in fillet and viscera of females and males of Atlantic salmon

$r = -0.82$, $p < .001$) and a non-significant trend in females ($t = -1.84$, $r = -0.22$, $p = .07$) (Tables 2 and 3, Figure 4).

Of the 1,195 *A. simplex* larvae found in the fillets, 697 larvae were found in the left fillet and 498 larvae in the right fillet (Table 5). The majority (93%) of the larvae were found in the hypaxial muscle sections anterior to the anus (L4-L6 and R4-R6). There were similar number of larvae in the left (122) and right (131) vent sections (L6 and R6) while there were approximately 68% more larvae in upper and lower left belly (534 in L4-L5) compared with the upper and lower right belly (324 in R4-R5). In total, only 84 larvae were found in the epaxial muscles and tail sections (L1-L3 and R1-R3) with similar numbers in the left (41) and right fillets (43). A 3.17 kg male salmon (fish no. 45 in Table 4) had an unusual high number of larvae (14) in the right tail (R3) section.

GLMM models controlling for fish ID showed that both the probability of having at least one larvae and the number of larvae in fillets were affected by side and fillet part (Table 6, Figure 5). The left side of fish was more likely to have at least one *A. simplex* larvae and had higher numbers of larvae than the right side. Fillet parts 1–3 were much less likely to have larvae and had fewer larvae than fillet parts 4–6 (the hypaxial muscle sections).

4 | DISCUSSION

The Atlantic salmon examined for the presence of *Anisakis* larvae in this study were sampled in the fjord outside the river mouth of Namsen, a large river located in mid-Norway. The fish had been feeding in the ocean for 1–4 years, and likely, they were all returning to their maternal river to spawn. One female salmon (fish no. 64 in Table 1) could be a repeated spawner based on the occurrence of parasitic gill maggots (*Salmincola salmoneus*) (Kusterle et al., 2013). This implies that it could have stayed for a period in freshwater between the feeding periods in the sea. However, as it appears likely that *Anisakis* larvae will survive the freshwater migration of Atlantic

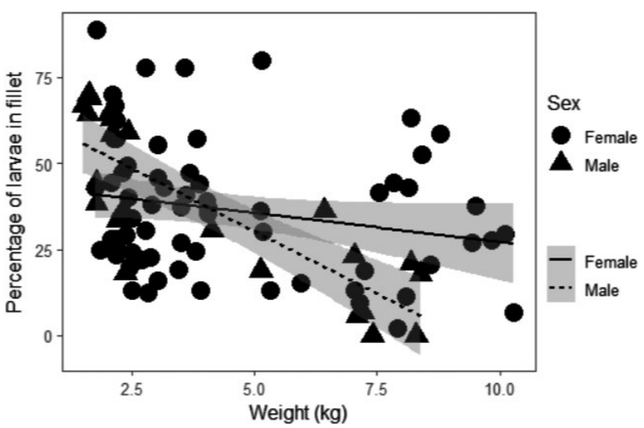

FIGURE 4 Correlation between weight of Atlantic salmon and percentage of *Anisakis simplex* larvae found in fillet relative to viscera for males and female fish

salmon (Wootten et al., 2010b), this assumed repeated spawner is merged with other three sea winter fish in the statistical tests.

When the fish were taken out of the bag nets and killed, they were kept in a boat at air temperature (about 15–20°C) for up to 1 hr before being put in a freezer. During this period, the internal temperature of the fish may have increased a few degrees Celsius and this could have resulted in increased activity and tissue migration of the nematode larvae. However, Karl et al., (2011) did not observe post-mortem migration of *Anisakis* larvae in Pacific salmon and we have assumed that this also applied to Atlantic salmon in our study.

The UV-press method (Karl & Leinemann, 1993; Levsen & Karl, 2014) could alternatively have been used by us for studying the presence and distribution of *Anisakis* larvae in the Atlantic salmon. This method is usually quicker than artificial digestion and is increasingly applied during systematic detection of nematode larvae in the flesh of fish, especially in large-scale scientific studies (Levsen et al., 2018). In our study, artificial digestion was chosen partly because the equipment needed already existed in the laboratory but also because it was considered challenging to press the internal organs and musculature of large Atlantic salmon specimens (>5 kg) in plastic bags sufficiently even if these fish sections could have been cut into smaller pieces. The UV method and the artificial digestion method have similar accuracy and sensitivity for the detection of nematode larvae (Gómez-Morales et al., 2018; Karl et al., 2011).

Mattiucci et al., (2017) listed numerous invertebrate and fish species as host for *A. simplex* larvae in the Northeast Atlantic Ocean. However, the family Salmonidae were not included in the list in spite well-documented occurrence of *A. simplex* in wild salmonids (Beverley-Burton & Pippy, 1978; Mo et al., 2010; Pippy, 1969; Senos et al., 2013; Wootten et al., 2010b). Furthermore, in studies using pop-up satellite archival tagged Atlantic salmon (Chittenden et al., 2013), the tags registered temperatures of 37°C for several days. The most likely explanation for the elevated temperature is that a whale had eaten the salmon and that the tag had passed the whale intestine and popped up after release through the anus (Chittenden et al., 2013). Hence, there is reason to claim that *A. simplex* can use Atlantic salmon as a fish host in its life cycle in a similar way as marine fish species.

4.1 | Abundance of *Anisakis simplex*

Anisakis simplex larvae were found in all Atlantic salmon examined in this study. Some previous studies found equal high prevalence (Beverley-Burton & Pippy, 1978; Senos et al., 2013; Wootten et al., 2010b), and as a consequence of their piscivorous predatory mode of life, the majority of Atlantic salmon in the Atlantic Ocean are infected (Beverley-Burton & Pippy, 1978). Interestingly, the number of *Anisakis* larvae per fish in more recent studies (Kent et al., 2020; Senos et al., 2013; Wootten et al., 2010b, this study) were much higher than the comprehensive study of Beverley-Burton and Pippy (1978) in the 1970s. The reason for the (seemly) increased intensity of *A. simplex* larvae in Atlantic salmon the last 4–5 decades can only

TABLE 5 Distribution of *Anisakis simplex* (*A.s.*) larvae in sections of the left (L) and right (R) fillets in 90 Atlantic salmon. The sectioning of the fillets is presented in Figure 1

Fish no.	Total *A.s.* in fillets	Total *A.s.* in left fillet	L1	L2	L3	L4	L5	L6	Total *A.s.* in right fillet	R1	R2	R3	R4	R5	R6
1	12	5	0	0	0	2	2	1	7	0	1	0	2	4	0
2	18	14	4	2	0	4	1	3	4	0	0	1	2	1	0
3	7	4	0	0	0	1	1	2	3	0	0	0	2	1	0
4	22	14	0	2	0	1	9	2	8	1	1	0	2	4	0
5	3	3	0	0	0	1	2	0	0	0	0	0	0	0	0
6	16	10	0	0	0	5	4	1	6	0	0	0	2	3	1
7	18	13	0	0	0	6	6	1	5	0	0	0	0	5	0
8	8	6	0	0	0	2	3	1	2	0	0	0	0	1	1
9	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
10	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0
11	9	6	0	0	0	2	4	0	3	0	1	0	2	0	0
12	8	3	0	0	0	1	2	0	5	0	0	0	1	2	2
13	7	5	1	0	0	2	1	1	2	0	0	0	1	1	0
14	7	5	0	1	1	0	3	0	2	0	0	0	1	0	1
15	17	8	0	0	0	2	6	0	9	0	0	0	5	3	1
16	7	6	0	0	0	3	3	0	1	0	0	0	0	1	0
17	4	0	0	0	0	0	0	0	4	0	0	0	0	1	3
18	6	2	0	0	0	0	2	0	4	0	0	0	0	4	0
19	24	14	1	0	0	5	4	4	10	0	0	1	5	2	2
20	22	15	0	1	0	2	8	4	7	0	0	0	3	4	0
21	12	7	0	0	0	4	3	0	5	0	0	0	3	2	0
22	9	4	0	0	0	2	2	0	5	0	0	0	1	3	1
23	5	1	0	0	0	0	1	0	4	0	0	0	2	1	1
24	7	7	0	0	0	5	2	0	0	0	0	0	0	0	0
25	8	4	0	1	0	3	0	0	4	0	0	0	0	3	1
26	4	2	0	1	0	0	1	0	2	0	0	0	0	1	1
27	4	2	0	0	0	2	0	0	2	0	0	0	1	1	0
28	11	4	0	0	0	2	1	1	7	1	0	0	3	2	1
29	41	27	1	1	0	9	8	8	14	0	0	0	4	5	5
30	13	10	0	0	0	7	2	1	3	0	0	0	1	1	1
31	8	6	0	0	0	2	4	0	2	0	0	1	0	1	0
32	8	4	0	0	0	1	2	1	4	0	0	0	0	3	1
33	7	4	0	0	0	3	1	0	3	0	0	0	0	0	3
34	18	9	0	0	0	5	4	0	9	0	0	0	1	7	1
35	15	11	0	0	1	7	2	1	4	0	0	0	0	2	2
36	9	6	1	0	0	2	1	2	3	0	0	0	1	1	1
37	14	4	0	0	0	1	2	1	10	0	3	0	3	4	0
38	17	14	0	0	0	6	5	3	3	0	0	0	1	0	2
39	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0
40	12	8	0	0	0	2	6	0	4	0	0	0	1	3	0
41	16	12	0	0	1	2	9	0	4	0	0	1	0	2	1
42	6	5	1	0	0	2	2	0	1	0	0	0	1	0	0

(Continues)

TABLE 5 (Continued)

Fish no.	Total *A.s.* in fillets	Total *A.s.* in left fillet	L1	L2	L3	L4	L5	L6	Total *A.s.* in right fillet	R1	R2	R3	R4	R5	R6
43	21	13	0	0	0	5	5	3	8	0	0	0	3	4	1
44	16	10	1	0	0	2	7	0	6	0	0	0	5	1	0
45	27	7	0	0	0	3	2	2	20	0	0	14	1	2	3
46	15	13	0	0	0	0	4	9	2	0	1	0	0	1	0
47	11	7	0	0	0	3	3	1	4	0	0	0	2	1	1
48	16	9	0	2	0	2	5	0	7	0	2	0	1	3	1
49	7	7	0	0	0	0	6	1	0	0	0	0	0	0	0
50	74	19	0	0	0	8	10	1	55	0	0	0	1	5	49
51	17	13	0	0	0	4	8	1	4	0	0	0	4	0	0
52	12	5	0	0	0	1	4	0	7	1	0	0	2	3	1
53	12	7	0	1	0	3	3	0	5	0	1	1	2	1	0
54	11	3	0	0	0	0	3	0	8	0	0	0	2	4	2
55	6	5	1	0	0	0	3	1	1	0	0	0	0	1	0
56	9	5	0	0	0	0	4	1	4	0	0	1	0	2	1
57	29	13	2	0	0	2	4	5	16	0	1	0	3	1	11
58	16	15	0	0	0	1	5	9	1	0	0	0	0	1	0
59	11	4	1	0	0	1	2	0	7	0	0	0	3	3	1
60	13	4	0	0	0	1	3	0	9	0	0	0	3	4	2
61	21	11	0	0	0	4	4	3	10	0	0	0	1	5	4
62	4	2	0	0	0	0	2	0	2	0	0	0	1	1	0
63	10	8	0	0	0	5	2	1	2	0	0	0	1	1	0
64	21	11	1	0	1	3	5	1	10	0	0	0	3	5	2
65	10	4	0	0	0	1	2	1	6	0	3	0	1	0	2
66	29	21	0	2	0	4	6	9	8	1	0	0	3	1	3
67	24	16	0	0	2	8	5	1	8	0	0	0	5	3	0
68	16	10	0	1	0	4	5	0	6	0	1	0	3	2	0
69	2	2	0	0	0	0	2	0	0	0	0	0	0	0	0
70	6	6	1	0	0	1	3	1	0	0	0	0	0	0	0
71	2	2	0	0	0	0	2	0	0	0	0	0	0	0	0
72	21	8	0	0	0	1	6	1	13	0	0	0	4	6	3
73	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
74	20	11	0	0	0	2	9	0	9	0	0	1	1	6	1
75	8	2	0	0	0	2	0	0	6	0	0	0	2	4	0
76	3	2	0	0	0	0	2	0	1	0	0	0	0	1	0
77	7	6	0	0	0	2	3	1	1	0	1	0	0	0	0
78	12	5	0	0	0	0	4	1	7	0	0	0	2	5	0
79	5	4	0	1	0	0	2	1	1	0	0	0	1	0	0
80	36	20	0	0	0	8	10	2	16	0	0	0	4	9	3
81	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
82	18	18	0	0	0	0	0	18	0	0	0	0	0	0	0
83	10	7	0	1	0	4	1	1	3	0	0	0	1	2	0
84	17	9	0	0	0	3	6	0	8	0	0	0	0	7	1
85	27	12	0	0	1	6	5	0	15	0	0	0	4	8	3

(Continues)

TABLE 5 (Continued)

Fish no.	Total *A.s.* in fillets	Total *A.s.* in left fillet	L1	L2	L3	L4	L5	L6	Total *A.s.* in right fillet	R1	R2	R3	R4	R5	R6
86	21	16	0	0	0	4	8	4	5	1	0	1	1	2	0
87	21	16	0	0	0	6	9	1	5	0	0	0	1	3	1
88	16	10	0	1	0	4	5	0	6	0	0	0	3	3	0
89	22	14	0	0	0	7	4	3	8	0	0	0	7	0	1
90	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Sum	1,195	697	16	18	7	222	312	122	498	5	16	22	132	192	131

be surmised. The Atlantic salmon may have shifted to prey species with higher *A. simplex* abundances, or they may feed in new areas with higher occurrence of *A. simplex* larvae. However, this could also reflect an increased number of specimens of whale species listed as the final hosts for *Anisakis simplex s.s.* (Mattiucci et al., 2017). In total, several hundred thousand specimens of the listed host whale species are now present along the Norwegian coast and in the open sea of the Northeast Atlantic Ocean (http://www.imr.no/) where the Atlantic salmon feeds (Thorstad et al., 2011). Similarly, an increased number of grey seal could explain the increased abundance of the nematode *Pseudoterranova decipiens* (Krabbe, 1878) in Baltic cod (Mehrdana et al., 2014). In a global perspective, Fiorenza et al., (2020) detected a significant 283-fold increase in *Anisaksis* spp. abundance in a meta-analysis of published papers in the period 1962–2015.

In addition to the generally increased intensities of *A. simplex* larva in Atlantic salmon, the increase in intensity may also be variable in different parts of the Atlantic Ocean. In 2015, 1SW Atlantic salmon sampled in the coastal water around Scotland had a mean of 259.9 *A. simplex* larvae (Kent et al., 2020). This is almost three times the number of larvae found in 1SW salmon sampled in 2009 in a southern Norwegian river (Senos et al., 2013) and about eight times higher than the number of *A. simplex* larvae in 1SW salmon sampled in middle Norway in 2013 (this study). Even if the salmon in the three studies were sampled in a few years apart, it could imply that the larval intensities increase most rapidly in the southern Atlantic Ocean. The intensity increase in Scottish 1SW Atlantic salmon could be related to climate change and immigration of more infected intermediate prey fish species and final host mammalian species (Kent et al., 2020).

As in a previous study (Beverley-Burton & Pippy, 1978), we found a positive correlation between the number of *A. simplex* larvae and the sea age of the Atlantic salmon. However, Beverley-Burton and Pippy (1978) observed more larvae in 3SW Atlantic salmon than in 4SW and 5SW Atlantic salmon in some sampling stations. A similar trend was observed in our study. As expected, the number of *A. simplex* increased from 1SW to 2SW Atlantic salmon, likely due to increased consumption of lager fish prey with more *A. simplex*. However, there were fewer larvae in 3SW males and in four 4SW females (when treated separately). One explanation could be the development of an inflammatory host response killing *A. simplex* larvae in the oldest fish. Alternatively, *A. simplex* larvae affect Atlantic salmon negatively in a way that the most infected specimens disappear from the population. For example, the most heavily infected Atlantic salmon may have a reduced swimming capacity and are thus more frequently eaten by a predator, for example a whale (see Barber et al., 2000).

4.2 | Tissue distribution of *Anisakis simplex*

Beverley-Burton and Pippy (1978) found 60.8% and 39.2% of 641 *A. simplex* larvae from the viscera and musculature, respectively, in 70 1SW Atlantic salmon. Senos et al., (2013) found the exact same

TABLE 6 Generalized linear mixed models (GLMM) on a) the probability of having one or more *Anisakis simplex* larvae modelled using a binomial distribution (1,080 fillet parts, 90 fish) and b) the number of larvae using a Poisson distribution (421 fillet parts, 88 fish) in relation to side and fillet part of Atlantic salmon

	Estimate	SE	Z	*p*
a) Binomial model				
(Intercept)	−2.46	0.31	−8.01	<.001
Side (right)	−0.43	0.17	−2.47	.0134
Fillet (2)	0.48	0.35	1.37	.17
Fillet (3)	−0.15	0.39	−0.39	.70
Fillet (4)	3.71	0.34	10.81	<.001
Fillet (5)	4.54	0.37	12.16	<.001
Fillet (6)	2.65	0.32	8.20	<.001
b) Poisson model				
(Intercept)	0.21	0.23	0.93	.35
Side (right)	−0.20	0.06	−3.44	<.001
Fillet (2)	0.12	0.28	0.42	.68
Fillet (3)	0.46	0.29	1.56	.12
Fillet (4)	0.80	0.23	3.50	<.001
Fillet (5)	1.02	0.23	4.51	<.001
Fillet (6)	0.74	0.23	3.21	.001

Note: Individual salmon identity was included as a random effect to control for non-independence of fillet parts within each fish.

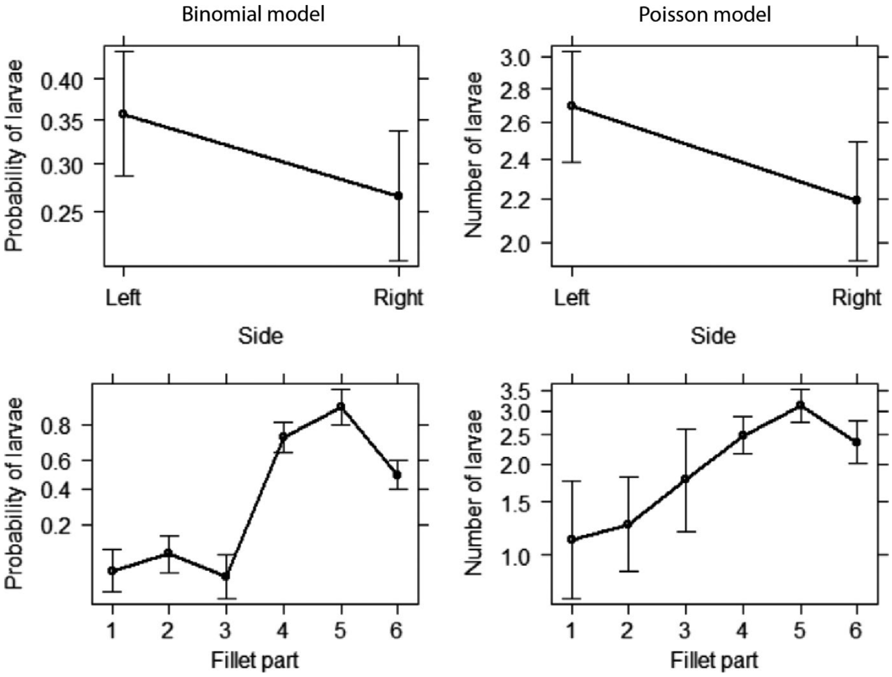

FIGURE 5 Depiction of modelled effects of Atlantic salmon *Salmo salar* sea age and weight on the probability of *Anisakis simplex* larvae (left, Binomial model) and the number of larvae (right, Poisson model) in relation to side (top) and fillet part (bottom) as estimated in the generalized mixed models (GLMM) in Table 5

distribution among 1524 *A. simplex* larvae in 17 1SW Atlantic salmon even if the abundance of larvae was more than ten times higher. A similar distribution of *A. simplex* larvae was found in the musculature (36.8%) in 1SW Atlantic salmon in our study.

Even if the number of *A. simplex* larvae increased with increasing weight and sea age of Atlantic salmon in our study, the percentage of *A. simplex* larvae in the musculature decreased. This implies that *A. simplex* larvae increasingly established in the viscera as the growing salmon become more infected. This could be explained by an increased larval migratory distance as the fish become larger, and possibly, only a few larvae were able to migrate the distance to the musculature. However, as suggested by Murphy et al., (2010), earlier migrating larvae could result in inflammatory responses that negatively affect the migration of larvae ingested later by the fish.

The majority (93%) of the *A. simplex* larvae in the musculature occurred in the hypaxial sections anterior to the anus. This was expected as most *Anisakis* larvae parasitize the ventral portion of the flesh surrounding the visceral cavity in marine fish species (Levsen et al., 2018).

A condition named red vent syndrome (RVS) has been described in returning wild Atlantic salmon (Beck et al., 2008). Large numbers of larval *A. simplex* were found in the perianal tissues, resulting in a red, swollen vent area (Beck et al., 2008; Mo et al., 2010; Noguera et al., 2009). The role of *Anisakis* larvae in this manifestation can only be surmised. In our study, a red vent was not visually observed in any of the fish and the number of *A. simplex* larvae in the perianal tissues (L6 + R6) was lower than the number of larvae in the hypaxial musculature including the belly flaps (L4-5 + R4-5). We are not aware of any cases of rent vent reported in Norway since late 2000s (Mo et al., 2010), and the red vent syndrome seems to have disappeared as quickly as it appeared, at least in Atlantic salmon migrating to Norwegian rivers. The high intensities of *A. simplex* larvae in 1SW Atlantic salmon in coastal water of Scotland explain the frequent occurrence of RVS symptoms in these fish (Kent et al., 2020). The eight times lower intensities in 1SW salmon in our study may explain that RVS symptoms are rarely reported in Atlantic salmon caught in bag net of Norwegian coastal water coast or by anglers in Norwegian rivers.

4.3 | The risk of humans developing anisakiasis

Third stage larvae (L_3) of the genus *Anisakis* are known for their zoonotic potential and are considered a human health hazard (Mattiucci et al., 2017), and especially *A. simplex* sensu stricto larvae as this is the most common species in the fish musculature (Suzuki et al., 2010). Thus, international regulations, for example EU No. 1276/2011, demand deep-freezing for at least 24 hr of any fishery product to be consumed raw or semi-raw, the so-called freezing requirement (Levsen et al., 2018). However, the documented absence of *Anisakis* larvae in muscle fillets of farmed Atlantic salmon (Angot & Brasseur, 1993; Deardorff & Kent, 1989; Levsen & Maage, 2016; Lunestad, 2003; Mo et al., 2014; Wootten et al., 2010a) has granted farmed Atlantic salmon an exemption from the freezing requirement. However, the presence of *A. simplex* in farmed Atlantic salmon runts (loser fish) has been described (Levsen & Maage, 2016; Mo et al., 2014). This exemption does not apply to wild Atlantic salmon where the presence of *Anisakis* larvae in the muscle fillets is well documented (Beverley-Burton & Pippy, 1978; Kent et al., 2020; Senos et al., 2013; Wootten et al., 2010b). Unfortunately, most people do not know the risk difference between eating raw or semi-raw farmed versus wild Atlantic salmon The increasing popularity for raw and semi-raw products such as sushi and sashimi has resulted in an increased use of own wild fish catches in such dishes, and at least in

Norway, fresh catches of Atlantic salmon are consumed raw on the river bank (personal observations). As a consequence, the exemption from the freezing requirement of farmed Atlantic salmon and other farmed fish (EFSA Panel on Biological Hazards (BIOHAZ), 2010) may unintentionally result in more cases of human anisakiasis. Thus, the authorities should improve their risk communication concerning anisakiasis and the difference when eating unfrozen raw or semi-raw farmed and wild salmonids and other fish species. Even if our study showed an increasing number of *A. simplex* larvae with increasing weight and sea age of the Atlantic salmon, the number of larvae per weight musculature decreased with increasing fish weight and sea age. Thus, eating a meal of raw or semi-raw small 1SW Atlantic salmon is at higher risk for anisakiasis than larger MSW salmon.

In conclusion, our work documents a very high prevalence of *A. simplex* sensu stricto larvae in adult Atlantic salmon returning to Norwegian rivers and that the intensity of such larvae increases with increasing weight and number of years of feeding in the sea. *A. simplex* larvae are commonly found in the musculature of Atlantic salmon. The consequences of these nematode larval infections in salmon, especially the musculature, on its behaviour and survival remain unknown. As live *A. simplex* larvae may cause anisakiasis in humans, consumption of raw or semi-raw wild Atlantic salmon is not recommended.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TAM designed the study. TAM and TTP examined the fish and were responsible for the digestion with assistance from a laboratory technician. FF was responsible for the genetic and statistical analysis. TAM wrote the manuscript with contributions from FF and TTP.

ETHICAL APPROVAL

The sampling of wild Atlantic salmon was approved by the county governor in Trøndelag, and the sampling was done by two fishermen with official permission to catch Atlantic salmon in bag nets.

DATA AVAILABILITY STATEMENT

All data on salmon and parasite occurrence that were compiled in this project are presented in tables in the manuscript.

ORCID

Tor Atle Mo https://orcid.org/0000-0002-2580-5679

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