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RESEARCH ARTICLE

Contracaecum osculatum and *Pseudoterranova* sp. in the liver of salmon (*Salmo salar*) from Polish marine waters

Katarzyna Nadolna-Ałtyn, Joanna Pawlak, Magdalena Podolska, Adam M. Lejk

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Abstract. Anisakidae nematodes, especially *Contracaecum osculatum*, *Anisakis simplex*, and *Pseudoterranova decipiens,* have dispersed throughout the Baltic Sea over the last decade. Despite the fact that salmon, *Salmo salar*, is a popular choice among consumers and therefore one of the most valuable Baltic fish species, information about the level of infection of salmon liver with these zoonotic nematodes is sparse. In 2020, a total of 120 salmon livers were inspected for the presence of parasites showing that 13% of salmon livers were infected with *C. osculatum*. Furthermore, a single *Pseudoterranova* sp. larva was detected in one salmon liver, representing a host-parasite system that has never previously been reported in the Baltic Sea.

Keywords: Anisakidae, Baltic Sea, nematodes

Introduction

Over the last decade, Anisakidae nematodes (especially *Contracaecum osculatum*, *Anisakis simplex*, and *Pseudoterranova decipiens*) are known to have dispersed throughout the Baltic Sea. An increase in both the prevalence and intensity of infection with *C. osculatum* has been recorded for cod, *Gadus morhua* L. (Haarder et al. 2014, Mehrdana et al. 2014, Nadolna and Podolska 2014, Horbowy et al. 2016). These parasites have also recently been recorded in fish species such as sprat, *Sprattus sprattus* (L.) (Zuo et al. 2016, 2017, Nadolna-Ałtyn et al. 2018), which previously was only rarely infected with Anisakidae nematodes. They have also even been recorded in species such as the great sandeel, *Hyperoplus lanceolatus* (Le Sauvage) (Nadolna-Ałtyn et al. 2017), which was previously free of them. *A. simplex* larvae parasitize herring, *Clupea harengus* L., which is then a source of infection for salmon, *Salmo salar* (ICES 2020).

The life cycles of *A. simplex*, *C. osculatum*, and *P. decipiens* are very similar, with marine mammals as their final hosts (McClelland et al. 1990, Klimpel and Palm 2011). However, there is some host specificity, and particular species of marine mammal are responsible for closing the life cycle of each parasite species. The final hosts for *A. simplex* are cetaceans, which in the Baltic Sea are represented by the harbor porpoise, *Phocoena phocoena* (Herreras et al. 2004); for *C. osculatum*, the final host is the grey seal, *Halichoerus grypus* (Fagerholm 1990), while for *P.*

K. Nadolna-Ałtyn [✉], J. Pawlak, M. Podolska
Department of Fisheries Resources, National Marine Fisheries Research Institute, Gdynia, Poland
E-mail: knadolna@mir.gdynia.pl

A. M. Lejk
Department of Logistics and Monitoring, National Marine Fisheries Research Institute, Gdynia, Poland

decipiens, it is the harbor seal, *Phoca vitulina* (Aspholm et al. 1995), and the grey seal (Hauksson 2011). In the final host, larval stage L4 transforms to the mature stage and fertilized eggs enter the aquatic environment in the host's feces. Transformation to larval stages L1, L2, or even L3 occurs in the egg (Køie and Fagerholm 1995). Subsequently L2- or L3-stage larvae are eaten by crustaceans, which play the role of intermediate host, where transformation from L2 to L3 can take place. Infected crustaceans are eaten by pelagic fish (e.g., sprat, herring), which in turn are eaten by predatory piscivorous fishes (e.g., cod, salmon). Marine mammals are the final link in the trophic chain; they feed on infected fish and thus become the definitive, i.e., final, host and the life cycle is completed (Køie and Fagerholm 1995, Klimpel et al. 2004, Mouritsen et al. 2010).

Anisakidae nematodes ingested by fishes have the ability to migrate from the digestive tract to surrounding organs or to muscles tissue. Each of the three parasite species analyzed in this study have different organ preferences, which can vary depending on the host species. *A. simplex* is often found in herring in the body cavity and on the surfaces of the viscera (Horbowy and Podolska 2001); in cod it is mostly recorded in muscles (Nadolna-Ałtyn et al. 2022); in salmon it was found in the muscles and viscera (Kent et al. 2020). *P. decipiens* prefer muscle tissue in both cod (Nadolna-Ałtyn et al. 2022) and salmon (Wotten et al. 2020). *C. osculatum* is found predominantly in the liver of cod (Nadolna and Podolska 2014) and salmon (Setyawan et al. 2019). Fish accumulate these nematodes throughout their lives. Humans can become infected after eating a meal of fish that contains viable parasites, which have zoonotic potential. Consumption of raw or unprocessed fish containing live Anisakidae nematodes can lead to anisakidosis. The symptoms of acute anisakidosis are nausea, diarrhea, vomiting, and intense abdominal pain (Hochberg et al. 2010, Ishikura et al. 1993). The appropriate preparation of infected fishes kills parasites (Wharton and Aalders 2002), but some allergenic proteins of anisakid nematodes are thermostable (Audicana et al. 2002, Moneo et al. 2005). Therefore, even dead parasites can be dangerous and can cause allergic symptoms.

Salmon is widely consumed by humans and is thus one of the most valuable Baltic fish species. Salmon fillets are prepared and served in many ways, including raw (as carpaccio or tartare), smoked, baked, and grilled. Salmon livers are eaten baked or as pâté. However, despite the fact that salmon is readily consumed, little is known about the level of infection with Anisakidae nematodes. There has only been one recent study, limited to 24 individuals from the coastline of the island of Bornholm, that describes endoparasitic helminths in Baltic salmon (Setyawan et al. 2019). Therefore, the aim of our study was to determine the level of infection with zoonotic Anisakidae nematodes in the liver of salmon from the Polish Exclusive Economic Zone (EEZ) in the Baltic Sea.

Materials and Methods

A total of 120 salmon (livers) from the Polish EEZ were collected over two seasons in 2020 for parasitological analysis: 52 fish at the beginning of the year (January-April) and 68 at the end (November-December). The distribution of samples according to sampling season is shown on the map (Fig. 1). Standard ichthyological analysis (fork length, body mass, sex, gonad developmental stage using the Maier (1908) scale, and age based on scale annuli) was conducted. Each fish was numbered individually, and the livers were collected during the above analysis and frozen for further investigation. Thawed livers were digested in artificial gastric juice (aqueous solution of pepsin and hydrochloric acid). After 24 h at room temperature, the livers were rinsed on a sieve under running tap water.

All nematodes were identified based on anatomo-morphological features as described by Fagerholm (1982) and Berland (1989). One of the most characteristic features distinguishing representatives of the genera *Anisakis*, *Contracaecum*, and *Pseudoterranova* is the anatomy of the digestive tract: *Anisakis* has a simple stomach without a caecum; the

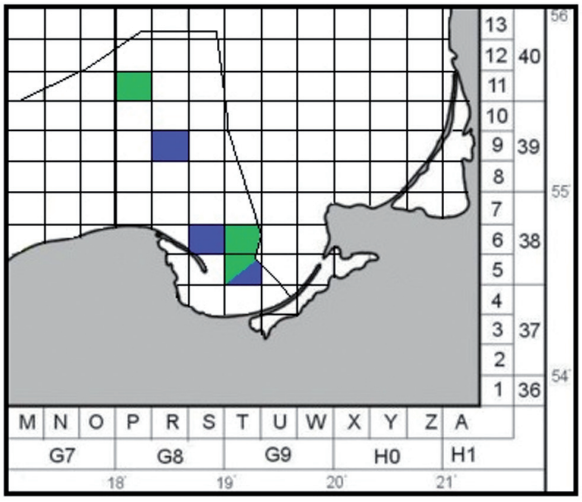

Figure 1. Fishing squares in the southern Baltic Sea where salmon were sampled for parasitological analysis of the liver (blue – sampling January-April; green – sampling November-December).

stomach of *Contracaecum* has two caeca (one is a ventricular appendage, the other is an intestinal caecum); the stomach of *Pseudoterranova* has one intestinal caecum directed towards the head. A second important characteristic is the position of the boring tooth in relation to the excretory pore: in *Anisakis*, the excretory pore is situated just behind the boring tooth; in *Contracaecum*, the anterior boring tooth is close to the opening of the excretory pore; *Pseudoterranova* is characterized by the anterior boring tooth being close to the opening of the excretory pore. A third characteristic (related with morphology of the larva) is the shape of the tail: *Anisakis* has a typical terminal mucron; *Contracaecum* has a conical tail with a rounded end; in *Pseudoterranova* the tip of the tail bears a small spine or mucron. Thus, differentiating Anisakidae larvae to the genus level (*Anisakis*, *Contracaecum*, and *Pseudoterranova*) is possible based on these anatomo-morphological features. However, it must be emphasized that *Anisakis, Contracaecum*, and *Pseudoterranova* are examples of sibling species complexes, and it is impossible to distinguish species in the complex purely using anatomo-morphological features. Nevertheless, species identification is possible using a genetic approach. For this reason a molecular investigation is necessary for taxonomic identification to the species level.

Parasitological descriptors were calculated following the definitions given by Bush et al. (1997). Prevalence is "the number of hosts infected with one or more individuals of a parasite species (or of a taxonomic group) divided by the number of hosts examined for that parasite species." The intensity (of infection) is "the number of individuals of a particular parasite species in a single infected host."

All parasites were subjected to molecular identification methods. The target of molecular analysis in all cases was the internal transcribed spacer 1 of ribosomal DNA (ITS-1 rDNA). Genomic DNA was isolated using a Sherlock AX kit (A&A Biotechnology) with mechanical lysis (shredding the tissue with a surgical blade). The amplification of ITS-1 was performed using the primers NC5 (forward), 5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3', and NC13R (reverse), 5'-GCT GCG TTC TTC ATC GAT 3' (Zhu et al. 2000, 2002). The polymerase chain reaction (PCR) mixture consisted of 25 µl 2x PCR Master Mix Plus High GC (ready-to-use PCR mixture containing Taq DNA polymerase, PCR buffer, $MgCl_2$ and dNTPs; A&A Biotechnology), 0.2 µl each primer (concentration 100 µM) and 5 µl DNA template, supplemented with deionized water up to 50 µl. The PCR conditions were as follows: 2 min at 94°C (initial denaturation) followed by 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 58°C for 30 s, and strand elongation at 72°C for 45 s, with a final extension step of 5 min at 72°C. PCR products were sequenced directly using standard procedures and amplification primers. Sequences were analyzed using GeneStudio TM Professional (GeneStudio, Inc., USA) and confirmed by a BLAST search of GenBank. One selected best quality sequence was deposited in GenBank, and the accession number is given in the Results section.

Results

The salmon sampled were between 63 cm and 107 cm in length. There were 41 males and 79 females. The fork length in both males and females was

Table 1
Biological parameters of the fish sampled with the prevalence and intensity of infection by sampling season

Season	N	Fork length (cm)		River age range (y)	Sea age range (y)	Prevalence (%)	Intensity of infection with *Contracaecum osculatum*
		mean	range				
January-April	52	84.9	69-107	1-4	2-4	17.31	1-9
males	19	87.1	73-105	1-4	2-4	26.32	1-9
females	33	83.7	69-107	2-4	2-4	12.12	1-9
November-December	68	76.1	63-107	2-4	1-3	10.29	1-8*
males	22	74.9	63-80	2-4	1-2	4.55	2
females	46	76.6	65-107	2-4	1-3	13.04	1-8*
Total	120	79.9	63-107	1-4	1-5	13.33	1-9

* plus one *Pseudoterranova* sp. larva

similar during each season, but fish caught at the beginning of the year had a slightly longer fork length than those sampled at the end of the year. The age of salmonid fish can be defined according to the time they spend in the river and in the sea. In our study, both the river age and the sea age ranged from one to four years. Details are presented in Table 1.

The prevalence of salmon liver infection with Anisakidae nematodes, which is presented by sex and season in Table 1, was 13% overall. In general, the prevalence was slightly higher in males (~14%) than in females (~12%). The intensity of salmon infection varied from one to nine nematodes per liver. In both seasons (Table 1), fish were infected with up to nine Anisakidae nematodes per liver, regardless of the sex of the fish (the exception being a single male salmon infected at the end of the year with two nematodes). However, most fish (56%) were infected with a single larva of *C. osculatum*, 25% with two, three, or four larvae, and ~19% with nine Anisakidae larvae

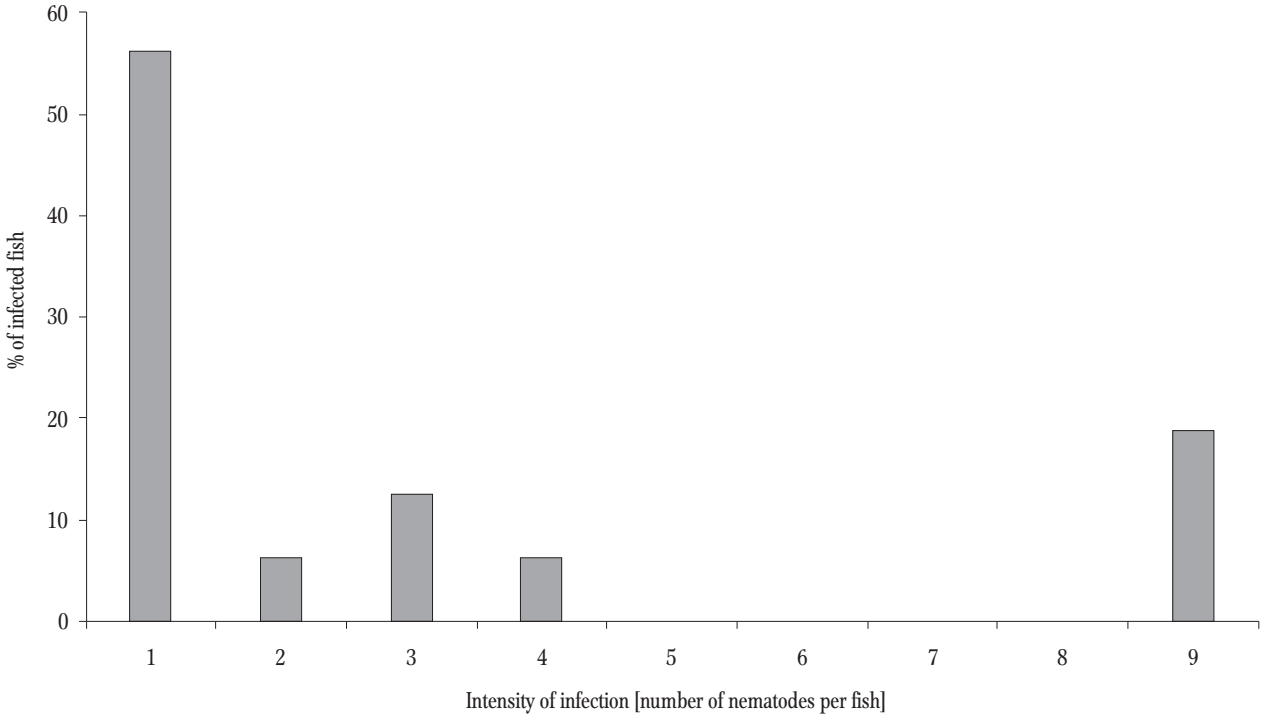

Figure 2. Proportion of fish with particular intensities of infection.

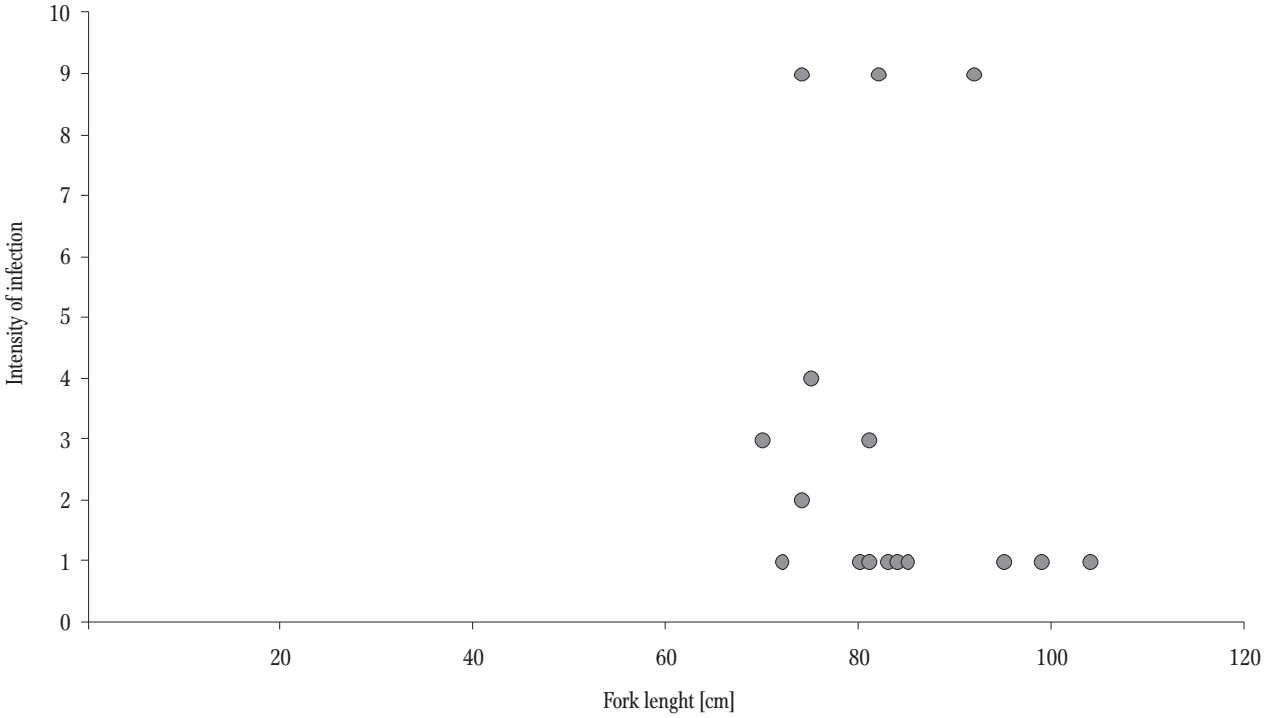

Figure 3. Intensity of infection vs fork length (cm) of the fish.

Table 2
Biological parameters of infected salmon (y - year, n - number)

Sampling month	Sampling area by fishing square	Fork length (cm)	Total body weight (g)	Sex	Gonad developmental stage	Age river.sea (y)	n *Contracaecum osculatum*	n *Pseudoterranova* sp.	n Anisakidae
January-April	R9	74	4240	female	2	2.2	9	0	9
January-April	R9	81	6325	female	3	2.2	3	0	3
January-April	R9	81	5185	female	3	2.2	1	0	1
January-April	R9	82	5175	male	2	3.2	9	0	9
January-April	R9	83	5805	male	2	3.2	1	0	1
January-April	R9	84	6495	male	2	3.2	1	0	1
January-April	R9	95	10580	female	3	3.3	1	0	1
January-April	R9	99	12370	male	2	3.3	1	0	1
January-April	R9	104	13800	male	2	3.4	1	0	1
December	P11	70	3330	female	2	3.1	3	0	3
December	P11	72	4470	female	3	2.1	1	0	1
December	P11	74	4190	male	2	4.1	2	0	2
December	P11	75	4300	female	2	3.1	4	0	4
December	P11	80	5545	female	3	3.1	1	0	1
December	P11	85	5950	female	3	3.3	1	0	1
December	P11	92	8340	female	3	4.2	8	1	9

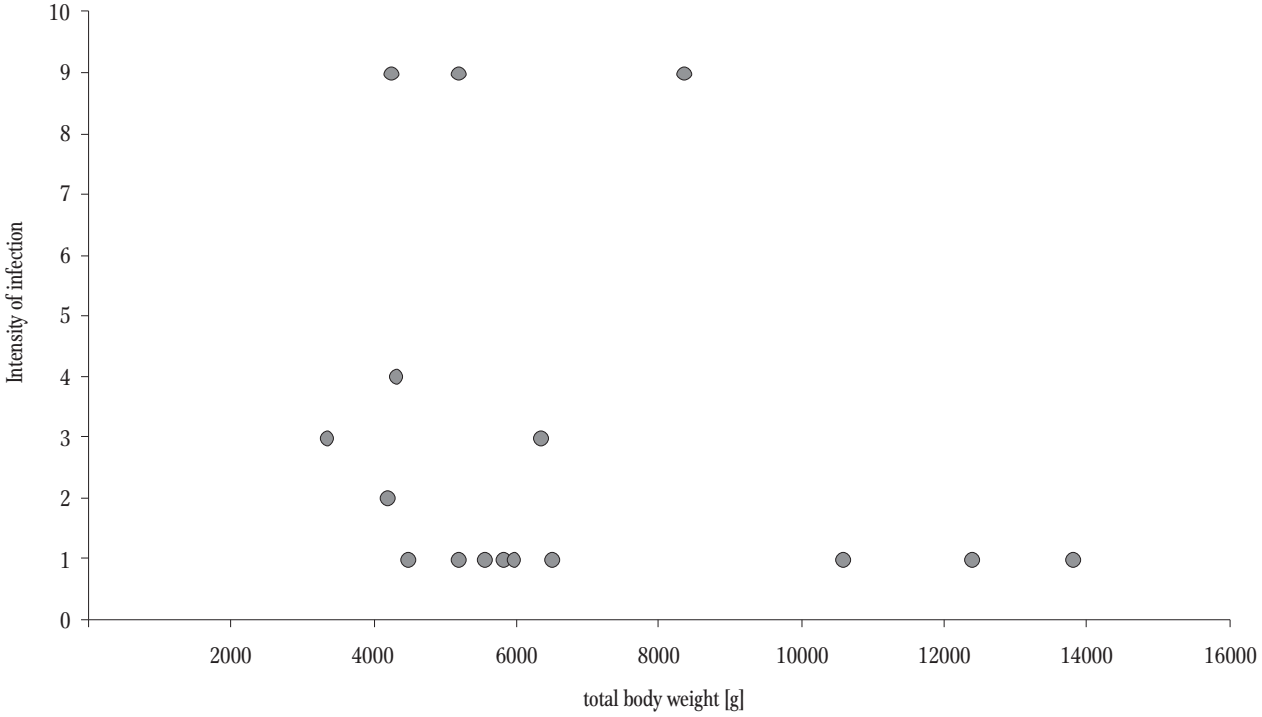

Figure 4. Intensity of infection vs total body weight (g) of the fish.

(Fig. 2). The intensity of infection was related neither to the fork length (Fig. 3) nor the total body mass (Fig.4) of the fish. Of the 16 fish infected with Anisakidae nematodes, ~37% were males and ~62% females (Table 2). At the beginning of the year infected fish were between 74 and 104 cm long, while at the end of the year infected salmon were only 70–80 cm long (Table 2). Even young fish (two river years and one year at sea) were found to be infected (Table 2). A single *Pseudoterranova* sp. larva was found in the company of eight *C. osculatum* larvae in a 92 cm-long female sampled in December. In both seasons, infected fish came from sampling areas located off-shore (fishing squares R9 and P11; see Fig. 1 and Table 2).

In total, 47 *C. osculatum* larvae and one *Pseudoterranova* sp. larva were detected. All larvae were initially identified on the basis of anatomo--morphological features. In the case of the single *Pseudoterranova* larva, a typical stomach with one caecum directed towards the head was observed. A characteristic *Pseudoterranova* larval tail, the tip of which bears a small mucron, was also identified. A boring tooth, situated close to the opening of the excretory pore, was visible.

To confirm the initial identification, all larvae were subjected to molecular analysis. In most cases, molecular analyzes confirmed the results obtained in microscopic examinations. One selected best quality sequence of *C. osculatum* was deposited in GenBank with accession number OP161017. The parasite was identified as *Contracaecum osculatum sensu lato*. Unfortunately, we were unable to establish a sequence for the *Pseudoterranova* sp. larva; although trace amounts of DNA were present in the sample, it was not possible to obtain PCR products because of DNA degradation.

Discussion

This study is the first attempt to describe the level of liver infection in salmon caught in Polish marine waters (southern Baltic Sea). Parasitological analyses revealed the presence of two of Anisakidae nematodes: Contracaecum (represented by C. osculatum) and

Pseudoterranova sp. In most cases, the initial taxonomic identification of *C. osculatum* was confirmed by molecular analysis. However, molecular identification was not successful for the single *Pseudoterranova* sp. larva. Salmon livers, including those containing nematodes, were collected during standard ichthyological analysis and then frozen. The relatively long process of obtaining parasites (freezing, additional digestion in artificial gastric juice) is likely to result in partial degradation of the DNA in a proportion of cases thus causing difficulties with molecular identification.

Infection of Baltic salmon livers with *C. osculatum* was previously described by Setyawan et al. (2019) for fish from the coastline of the island of Bornholm (45.8% prevalence; intensity 1-4); however, only 24 individuals were analyzed. A *Pseudoterranova* sp. larva in the liver of Baltic salmon was detected for the first time in this study.

Salmon is a diadromous fish that breeds in the rivers of the catchment areas of the Baltic Sea. However, fish from most of the Baltic salmon stocks migrate to feeding areas located in the central and southern parts of the Main Basin (Karlsson and Karlström 1994, Jacobson et al. 2020, Jones et al. 2022). Salmon is a piscivorous top predator (next to marine mammals and cod). It preys mainly on sprat, *S. sprattus*; herring, *C. harengus*; and three-spined stickleback, *Gasterosteus aculeatus* L., but representatives of Ammodytidae have also been recorded in the salmon diet (Christensen 1961, Christensen and Larsson 1979). Occasionally salmon have also been reported to take European perch, *Perca fluviatilis* L.; European smelt, *Osmerus eperlanus* L.; great sand eel, *Ammodytes* sp.; common bream, *Abramis brama* (L.); and viviparous eelpout, *Zoarces viviparus* (L.) (Karlsson et al. 1999, Hansson et. al. 2001). Although analyzed by many authors (for example Karlsson et al. 1999, Hansson et al. 2001, Salminen et al. 2001, Skóra and Haluch 2005), the most recent results show that the diet of salmon has not changed markedly since these earlier studies, with the predominant prey among fish being sprat, three-spined stickleback, and herring and among invertebrates *Mysis mixta* and *Gammarus* sp. (Nadolna-Ałtyn et al. 2022).

Prey are not only a source of nutrients, but they can also be a source of infection with parasites. The infection of Baltic sprat with *C. osculatum* has been documented (Zuo et al. 2016, 2017, Nadolna-Ałtyn et al. 2018), while herring is commonly infected with *A. simplex* and the presence of *C. osculatum* is also reported (Rodjuk 2014). *Gammarus* sp. infected with *C. osculatum* was found in the stomach of cod caught in Polish sea waters (Pawlak et al. 2019).

The widespread dispersion of Anisakidae nematodes in the Baltic Sea over the last ten years is related to the increasing number of marine mammals in the area, particularly the grey seal, *H. grypus*, which is well-documented as a final host in the life cycles of *C. osculatum* (Fagerholm 1990) and *P. decipiens* (Hauksson 2011).

The presence of nematodes in the liver negatively affects the condition of fish, as proven for Baltic cod (Mehrdana et al. 2014, Horbowy et al. 2016, Ryberg et al. 2020, 2022, Mohamed et al. 2020), and *C. osculatum* is likely to have a similarly negative impact on the salmon it infects. However, further studies on a larger group of fish are needed to assess the effects of the Anisakidae parasites on salmon condition. Salmon is an anadromous fish, which does not take prey during spawning migrations. The reservoir of energy deposited as lipids during the sea feeding period allows the fish to survive the migration back to their home rivers for reproduction. Over the spawning run, salmon reduce their preying activities and stop feeding for approximately four months before spawning (Vourinen et al. 2014). During this pre-spawning migration period, the fish rely mainly on their energy reserves (fatty acids) in visceral and tissue lipid stores (Sargent et al. 2002, Tocher 2003, Vourinen et al. 2020). In salmonid species before spawning, the muscular lipid content can decrease by 40–60%, and the visceral lipids by up to 70% (Corraze and Kaushik 1999). The low level of reserve material weakens the condition of the fish and might negatively affect their ability to migrate and reproduce.

When zoonotic nematodes are present in salmon fillets or livers, there is a risk to consumers of acute anisakidosis or allergy caused by eating infected raw

or underdone fish products. Indeed, one case study in Poland described anisakidosis probably caused by consumption of raw salmon meat (Kołodziejczyk et al. 2020). Strom et al. (2015) claim that *C. osculatum* might also be a zoonotic pathogen.

Similar studies should be conducted on a larger group of fish, to determine whether the presence of parasitic nematodes affects the condition of the salmon. To better understand how salmon become infected, a food-content analysis should be conducted to identify possible vectors of infection. In this study, only livers were analyzed for the presence of nematodes, but the most important fish parts from the consumer point of view are fillets. Therefore, studies on the presence of nematodes in salmon muscle should be conducted.

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ORCID iD

Katarzyna Nadolna-Ałtyn https://orcid.org/0000-0001-5507-2595
Joanna Pawlak https://orcid.org/0000-0002-4684-9670
Magdalena Podolska https://orcid.org/0000-0002-3303-3787
Adam M. Lejk https://orcid.org/0000-0002-1648-4308

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