

Research Article

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In situ evidence of the role of *Crangon crangon* in infection of cod *Gadus morhua* with nematode parasite *Hysterothylacium aduncum* in the Baltic Sea

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Abstract

Cod was one of the most important fish species in the Baltic Sea, but its condition is deteriorating for several reasons, including an increasing parasite burden. The aim of this study was to determine the source of infection of Baltic cod with parasites by examination of invertebrates found *in situ* in the cod stomach. A total of 1681 cod were sampled during four research cruises in the southern Baltic Sea in 2012, 2013 and 2014 and the composition of their diet was analysed. Each prey item from cod stomach was identified to the lowest possible taxonomic level and a parasitological analysis of all invertebrates collected was performed. *Crangon crangon*, *Saduria entomon* and *Mysis mixta* were the most commonly represented invertebrates among food items. *Hysterothylacium aduncum* was found only in *C. crangon*. This host–parasite system is reported here for the first time *in situ* in the stomach of cod from the Baltic Sea, confirming the role of *C. crangon* in cod infection with *H. aduncum*.

Introduction

Baltic cod (*Gadus morhua*) is one of the most commercially exploited fish species and is a popular food in several countries. Cod concentrates in deeper waters for spawning and migrates to the water column or shallow areas to feed (Bagge *et al.*, 1994). The dietary preferences of cod, which is a predatory fish throughout most of its life, depend on its ability to catch and eat specific prey species. Young cod mostly occur near the coast, in shallow water, and feed on invertebrates, especially Crustacea (e.g. *Crangon crangon*, *Mysis mixta* and *Gammarus* sp.). Older cod migrate to deeper, offshore waters where they prefer to eat fish (*Clupea harengus* and *Sprattus sprattus*) and larger invertebrates (*Saduria entomon*). Thus, the variability in the cod diet reflects the age of the fish and the biodiversity of prey species in the habitats occupied. Pachur and Horbowy (2013) revealed that a shift in dietary composition can be observed as cod reach between 30 and 40 cm in length. This shift in the cod's diet has consequences for the risk of infection with different parasite species.

The ecological status of the Baltic Sea is deteriorating and as a consequence the state of the cod population and the condition of individual cod grow progressively worse. This might be due to low availability of fish prey in areas where cod feed (Eero *et al.*, 2012); a lack of benthic prey because of lower dissolved oxygen or changes in salinity (Conley *et al.*, 2009; Carstensen *et al.*, 2014); intensive exploitation of resources, which negatively influences fish stocks (Lindegren *et al.*, 2009) and increasing infection with nematode parasites (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Horbowy *et al.*, 2016). The Baltic cod population has decreased drastically over the years, and the International Council for the Exploration of the Sea (ICES) has advised fisheries focused on cod in the Baltic Sea to cease fishing in 2020 (ICES, 2019).

Studies conducted on Gadiformes fish, specifically the burbot (*Lota lota*), by Valtonen and Julkunen (1995) revealed that the type of diet was a key factor determining the risk of infection with particular species of parasite. Cod can be intermediate, paratenic or definitive hosts for a large number of parasite species (Hemmingsen and MacKenzie, 2001), and the parasite fauna of cod differs depending on the size of individual fish (Zuo *et al.*, 2016). In the Baltic Sea, the dominant group of parasites in small cod is acanthocephalans, especially *Echinorhynchus gadi*, which occur in the digestive tract (Pilecka-Rapacz and Sobocka, 2004). In larger cod, the most abundant parasites are nematodes, particularly *Contraecaecum osculatum* (Szostakowska *et al.*, 2005; Nadolna and Podolska, 2014; Mehrdana *et al.*, 2014) and occasionally *Anisakis* sp. (Nadolna and Podolska, 2014), which occupy the liver; rarely, *Pseudoterranova* sp. and *Anisakis* sp. accumulate in the muscle tissue (Mehrdana *et al.*, 2014). *Hysterothylacium* sp. occurs mainly in the digestive tract of larger cod. In spite of the fact that the parasite fauna of cod from the Baltic Sea is well known and has been studied by several authors (Myjak *et al.*, 1994; Buchmann, 1995; Mellergaard and Lang, 1999; Perdiguero-Alonso *et al.*, 2008; Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Nadolna and Podolska, 2014; Zuo *et al.*, 2016), the life cycles of Baltic cod parasites are described only in general.

Cod infection with the nematode parasite *Hysterothylacium* sp., as well as its basic life cycle, is documented for the Baltic Sea, but little is known about the crustacean species that play the

role of intermediate host. The aim of this study was to determine the source of infection of Baltic cod with parasites by examination of invertebrates found *in situ* in cod stomach.

Materials and methods

The research material (cod stomachs) was collected during four research surveys in November (Q4) 2012 and 2013; as well as in February (Q1) 2013 and 2014. In total, 1681 cod stomachs were sampled in the Polish Exclusive Economic Zone, southern Baltic Sea. Ichthyological analysis of each individual cod was performed on board the survey ship. The stomachs were frozen at -20°C for further food content analysis in the laboratory on land. Analysis of the cod diet was performed: prey items found in stomachs were sorted and classified to the lowest possible taxonomic level (Żmudziński, 1990; Hayward and Ryland, 1995). All invertebrates were collected, counted and analysed one by one for the presence of parasites. The organisms found had decomposed to different extents: some were partly digested, whereas others were not macerated at all. Well-preserved invertebrates were digested in the laboratory in artificial gastric juice (aqueous solution of pepsin and 35–38% HCl) to expose any parasites in the body cavity. This treatment increased the transparency of prey organisms and improved the level of detection of parasites. All parasites observed were collected and examined under the stereomicroscope for taxonomic identification on the basis of anatomomorphological features (Fagerholm, 1982; Berland, 1989). Infection with parasites is described according to the prevalence and intensity of infection (Bush *et al.*, 1997).

To confirm taxonomic identification, molecular analysis of parasites was performed. Analysis was conducted according to Zhu *et al.* (1998) and involved the amplification and sequencing of the ITS-1 (Internal Transcribed Spacer) region of rDNA. Polymerase chain reaction (PCR) products were sequenced directly using standard procedures and amplification primers. DNA was isolated using a Genomic Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. Amplification 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'. The reaction mixture consisted of 25 μL PCR Master MixPlus 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 (stock concentration 100 μM), 20 μL DNA template and supplemented with deionized water up to 50 μL . The PCR reaction conditions were as follows: 2 min at 94°C (initial denaturation) followed by 35 cycles of 30 s at 94°C , 30 s at 60°C , 30 s at 72°C and a final extension step of 5 min at 72°C . Some DNA fragments obtained as a result of the amplification reaction were purified using a Gel-Out Concentrator Kit (A&A Biotechnology). PCR products were eluted with sterile water. Sequences were analysed using software CLC Workbench and GeneStudio and confirmed by a BLAST search of the GenBank.

Results

The food content analysis revealed 15 467 invertebrates in cod stomachs (Table 1). The dominant (the most numerous) species were Crustacea, especially *C. crangon*, *S. entomon*, *M. mixta*, *Gammarus* sp.; the polychaete *Byligides sarsi* was also frequently observed. Parasitological analysis of the invertebrates found in cod stomachs revealed the presence of *Hysterothylacium* sp. L3 larvae, but only in the decapod *C. crangon* (Fig. 1). The first microscopic investigation of *C. crangon* was insufficient, and additional digestion in artificial gastric juice was necessary to determine the presence of nematodes in the body cavity. Among the

4731 *C. crangon* examined, parasites were found in nine individuals: five in the sample from November 2012; one from February 2013; two from November 2013 and one from February 2014 (Table 2). On the basis of anatomo-morphological features, all the above parasites were identified as *Hysterothylacium* sp. Therefore, the prevalence of *C. crangon* infection with *Hysterothylacium* sp. was 0.0027% in November 2012; 0.0005% in February 2013; 0.0067% in November 2013 and 0.0018% in February 2014. The mean prevalence of infection was 0.0029%. The intensity of infection was 1 in every case.

Molecular analysis identified seven parasites as *Hysterothylacium aduncum*; two individuals were impossible to verify by DNA sequencing. The long process of obtaining parasites from under the carapace of crustaceans (digestion in stomach, freezing, additional digestion in artificial gastric juice) is likely to result in the partial degradation of the DNA in a significant proportion of cases and cause difficulties with molecular identification. Where parasites were successfully identified, the sequence similarity was 98.21–100% with *H. aduncum* compared to examples registered in the GenBank. Examples of the sequences obtained have been deposited in the GenBank (accession no. MW506285, MW506286, MW506287, MW506288 and MW506289).

Table 3 shows all parasites found in cod stomachs near to, but not within, food items (excluding samples from 2012, where they were not collected). The nematode *Hysterothylacium* sp. and the acanthocephalan *E. gadi*, as well as representatives of Trematoda, were found.

Discussion

Several recent studies have revealed a remarkable increase in the prevalence of cod infection with Anisakidae nematodes (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Nadolna and Podolska, 2014; Horbowy *et al.*, 2016; Zuo *et al.*, 2016). The negative effect of such an increase in the intensity of infection on the condition of fish has also been reported (Horbowy *et al.*, 2016). The parasite fauna of Baltic cod is well known (Myjak *et al.*, 1994; Buchmann, 1995; Møllergaard and Lang, 1999; Perdiguero-Alonso *et al.*, 2008; Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Nadolna and Podolska, 2014; Zuo *et al.*, 2016). Invertebrates, which represent important basic food items for Baltic cod in their early stages of development, are thought to be the first intermediate host and transmitter of pathogenic nematodes to fish (Bagge *et al.*, 1994; Horbowy *et al.*, 2016; Engelhardt *et al.*, 2020). The role of particular invertebrate species in the life cycles of specific parasites is not precisely defined. Clearly, a high-quality diet is essential for healthy fish development.

Similar to the research conducted by Pachur and Horbowy (2013), in the current study, the dominant invertebrate food items in cod stomach were Malacostraca, especially *C. crangon*, *S. entomon*, *M. mixta*, *Gammarus* sp. and Polychaeta *B. sarsi*. The presence of the parasite in the brown shrimp suggests that this invertebrate is not only a source of nutrients, but might also be a route of infection with parasites. However, parasites were found only in the brown shrimp, *C. crangon* (Decapoda), which frequently occurs in offshore sandy and sandy-muddy habitats in the Baltic and North Seas, and also along the north and west coasts of Europe and the American coastal waters of the North Atlantic (Żmudziński, 1967). Brown shrimp is a migratory species: in the autumn, when temperatures decrease, it migrates into deeper waters, returning to shallower waters in the spring (Żmudziński, 1961). Therefore, it is accessible to both large demersal cod in the colder part of the year and to small cod individuals present in shallow waters. In the Polish waters of the southern Baltic Sea, *C. crangon* reaches 55 (male) to 70 (female) mm in size (Szaniawska, 1991), making it easily

Table 1. Dominant invertebrate species among food items in cod stomachs (number of individuals)

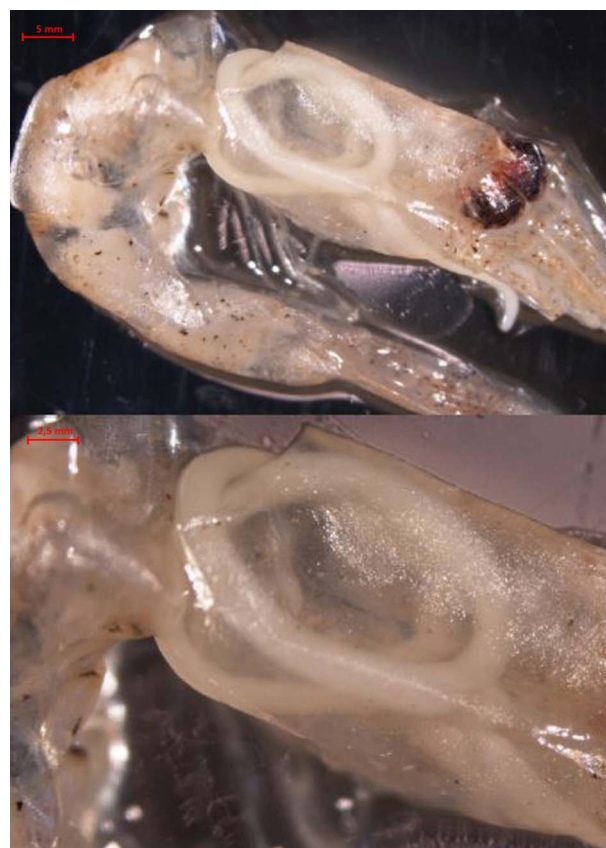
	2012 Q4	2013 Q1	2013 Q4	2014 Q1	Sum
No. of cod stomachs analysed	363	380	394	544	1681
<i>Crangon crangon</i>	1843	2030	299	559	4731
<i>Saduria entomon</i>	518	282	350	687	1837
<i>Mysis mixta</i>	184	990	1927	1357	4458
<i>Bylgides sarsi</i>	82	67	486	161	796
<i>Gammarus</i> sp.	169	184	209	534	1096
Other species	325	479	583	1162	2549
Total	3121	4032	3854	4460	15 467

available even to small cod; consequently, it is a common item in the cod diet (Pachur and Horbowy, 2013). The role of *C. crangon* as a transmitter of the nematode parasite *Anisakis simplex* to Baltic cod has been described by Pawlak *et al.* (2019). The results presented in this report are the first *in situ* confirmation that *C. crangon* may be a route for cod infection with *H. aduncum*.

In this study, *C. crangon* was found to be infected with L3-stage *H. aduncum* larvae. Genetic identification confirmed the results of anatomo-morphological analysis. To my best knowledge, this is the first evidence for this host–parasite system in the Baltic Sea. The prevalence of *C. crangon* infection with *Hysterothylacium* sp. was 0.0027% in November 2012; 0.0005% in February 2013; 0.0067% in November 2013 and 0.0018% in February 2014; the intensity of infection was 1 in all cases. Although the prevalence of infection is low, *C. crangon* is an important food item in the cod diet, and therefore this invertebrate may play a role as a transmitter of parasites to cod.

The nematode *Hysterothylacium* sp. (mainly *H. aduncum*), a member of the Raphidascarididae family, is a common parasite of marine fish throughout the world (Andersen, 1993; Rello *et al.*, 2008; Knoff *et al.*, 2012; Moravec *et al.*, 2012; Kong *et al.*, 2015; Morsy *et al.*, 2015; Shamsi *et al.*, 2015; Shamsi *et al.*, 2016; Shamsi, 2017; Ghadam *et al.*, 2018; Roca-Geronès *et al.*, 2018) and is the most frequently occurring parasite in invertebrates acting as intermediate hosts: it has been reported in 70 different invertebrate species (Lick, 1991). *Hysterothylacium aduncum* (Rudolphi 1802) has a circumpolar distribution in the Northern hemisphere (Deardorff and Overstreet, 1981) and has been found in the north-west Atlantic (Marcogliese, 1996), the North Sea and the Baltic Sea (Lick, 1991; Klimpel and Ruckert, 2005), but also in the Mediterranean Sea (Dural *et al.*, 2011; Abdel-Ghaffar *et al.*, 2015), the Black Sea (Pekmezci *et al.*, 2013) and the waters around Japan (Moravec and Nagasawa, 1986; Kong *et al.*, 2015). In the southern hemisphere, it has been found among other locations in the south-west Atlantic (Navone *et al.*, 1998), and around Australia (Shamsi *et al.*, 2015, 2018).

In general, in the *H. aduncum* life cycle, invertebrates play the role of intermediate hosts and fish are the final host. Natural infection with larval *H. aduncum* has been documented in seven phyla of both benthic and planktonic invertebrates in the north-western Atlantic (Norris and Overstreet, 1976; Marcogliese, 1996). Third-stage larvae of *H. aduncum* have been obtained from *C. crangon* in the Ythan estuary (Scotland)

**Fig. 1.** *Crangon crangon* infected with *Hysterothylacium aduncum* (photo: J. Pawlak).

(Gibson, 1972). In the Canadian Bras d'Or Lake, *H. aduncum* uses a variety of intermediate hosts including the mysids *Neomysis americana* and *M. stenolepis* and the chaetognath *Sagitta elegans*, as well as mixture of zooplankton: calanoid copepods, crab zoea and megalops and euphausiid larvae (Jackson *et al.*, 1997). *Hysterothylacium* sp. has been reported by Lick (1991) in several invertebrate species from the North Sea and Baltic Sea (studies limited to German coastal waters), including *Acartia bifilosa*, *Eurytemora affinis*, *Temora longicornis*, *Pseudocalanus elongatus* and *M. mixta*. Infection of gammarid species including *Gammarus locusta*, *G. salinus* and *G. zaddachi* with *Hysterothylacium* sp. in the same area has been noted by Lick (1991) and Zander *et al.* (2000). The same nematode parasite has also been recorded in representatives of Calanoida (Svendson, 1990; Marcogliese, 1995), especially in *Hyperia galba* and *Idotea* spp. from the North Sea (Klimpel and Ruckert, 2005) and *Neomysis. integer* from both the North Sea and German coastal waters of the Baltic Sea (Lick, 1991; Klimpel and Ruckert, 2005). Pawlak *et al.* (2018) found *S. entomon* infected with *H. aduncum* *in situ* in cod stomach, which was the first evidence of such a host–parasite system, similarly to the *C. crangon* and *H. aduncum* system in the Baltic Sea presented here.

Experimental research described in the literature confirms that the role of intermediate host may be served by invertebrates, for example, Crustacea, Polychaeta, Ctenophora, Echinodermata, Chaetognatha and Mollusca (Køie, 1993; Münster *et al.*, 2015). The life cycles of *Hysterothylacium* sp. and potential intermediate hosts were also studied after experimental infection of *T. longicornis* (Køie and Fagerholm, 1995) and calanoid species (Hurst, 1984; Køie, 1993), especially *N. integer* (Køie and Fagerholm, 1995).

In the Baltic Sea, the life cycle of *H. aduncum*, and in particular which invertebrate species serve as intermediate hosts for this parasite, has been described only in general terms. The marine

Table 2. *C. crangon* infected with *Hysterothylacium aduncum*/*Hysterothylacium* sp. nematodes in stomachs of Baltic cod

	2012 Q4	2013 Q1	2013 Q4	2014 Q1	sum
No. of cod stomachs with <i>C. crangon</i>	197	84	76	76	433
Total no. of <i>C. crangon</i>	1843	2030	299	559	4731
No. of <i>C. crangon</i> infected with <i>H. aduncum</i> / <i>Hysterothylacium</i> sp.	5	1	2	1	9
Prevalence of infection (%)	0.0027	0.0005	0.0067	0.0018	

Table 3. Parasites found in stomachs of Baltic cod

	2012 Q4	2013 Q1	2013 Q4	2014 Q1	Sum
Nematoda: <i>Hysterothylacium</i> sp.	No data	23	19	19	61
Acanthocephala: <i>Echinorhynchus gadi</i>	No data	26	4	71	101
Trematoda	No data	4	5	1	10

environment is changing and new species that act as intermediate hosts might appear. It is known, however, that the eggs of this nematode (which contain developed larvae) can be ingested by both benthic and pelagic crustaceans. Eggs hatch in the intestine of these invertebrates and the parasite larvae migrate to the haemocoel of the intermediate host. Larger invertebrates can play the role of second intermediate hosts (Køie, 1993).

Pawlak *et al.* (2018) revealed that moulting and transformation from L4 larva to the adult nematode (*H. aduncum*) might take place inside invertebrate hosts (*S. entomon*). Iglesias *et al.* (2002) conducted *in vitro* cultivation of *H. aduncum* from L3 to egg-laying adults and described the conditions (temperature = 13°C; pH = 4; 5% CO₂ in the growth atmosphere, etc.) for optimal development and survival of these nematodes. The authors proved that if the medium was supplemented with pepsin, all larvae reached the adult stage. Similar conditions were used for *in vitro* cultivation of L3-stage *H. aduncum* larvae obtained from the fish host through a complete developmental cycle of the parasite to L3 larvae hatched from eggs obtained during the experiment (Adroher *et al.*, 2004).

In the life cycle of *H. aduncum* the final hosts are fish. The larval stages live in different tissues of several fish species and in numerous invertebrate species (Norris and Overstreet, 1976; Hurst, 1984; Marcogliese, 1996). The parasites enter the fish with food and are able to penetrate the stomach wall of the fish to get to the body cavity and internal organs, e.g. liver (Myjak *et al.*, 1994). Sexually mature adult individuals of *H. aduncum* are often found in the digestive tract of fish (Deardorff and Overstreet, 1981), such as eel-pout and Atlantic cod (Jackson *et al.*, 1997), including the Baltic Sea (Køie, 1993). *Hysterothylacium* sp. has also been reported in the Baltic in flatfishes (Køie, 1993), eel *Anguilla anguilla* (Køie, 1993), sea trout *Salmo trutta trutta* (Unger and Palm, 2016), sprat (Skrzypczak and Rolbiecki, 2015), sticklebacks (Køie, 1993) and Gobiidae fish (Zander *et al.*, 1993, 1994; Zander, 2004). Gadoids are considered to be the main final hosts for *Hysterothylacium* sp., however (Berland, 1961). Therefore *C. crangon* might also be the source of infection with this parasite for other listed fish species in the Baltic Sea that feed on the invertebrate.

The emergence of new intermediate hosts is an interesting development, to which the changing climate may be a contributory factor. Rokicki (2009) noted that, in general, environmental changes affect the occurrence and abundance of parasites either directly by their influence on the free-living larval stages of parasites or indirectly by their effect on the respective hosts (mainly invertebrate). This problem has already been noted in several parts of the world, such as Australia, where environmental changes

have negatively impacted the survival of early-stage parasite larvae in their first intermediate hosts. The absence of Anisakis larvae in fish collected in this area shows the importance of the role of zooplankton and crustaceans in the food chain and in the ecosystem more generally (Shamsi *et al.*, 2018). In Canada, the limited availability of specific food components may be one reason for the decreasing number of parasites in fish (Khan and Chandra, 2006).

In this study, the parasites *Hysterothylacium* sp., *E. gadi* and representatives of Trematoda were found in the stomachs of cod adjacent to food items. These parasites might therefore be present in the body cavity of prey before digestion in the stomach, which could be a source of infection.

It must be emphasized that when cod are caught in a particular area it does not unequivocally indicate that parasites found in its stomach or in its food items were obtained by the fish in the same area. Cod is a migratory species and the adult cod can migrate up to 1000 km (Saulamo and Neuman, 2002) without clear spatial or temporal distribution patterns (Aro, 2000).

In summary, to my best knowledge, *H. aduncum* has been reported in the Baltic Sea in *C. crangon* for the first time and this also represents the first report of this host–parasite system found *in situ* in cod stomach. Because *C. crangon* plays an important role in the food composition of cod, this invertebrate is likely to be an intermediate host in the life cycle of *H. aduncum* in the Baltic Sea. The results of this research are important for the development of the parasitology, fish biology and ecology of the Baltic Sea, in particular for an improved understanding of the function of individual components of the food web in the transmission of cod parasites.

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Conflict of interest. The author declares none.

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