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NOTE

Host size-dependent anisakid infection in Baltic cod *Gadus morhua* associated with differential food preferences

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ABSTRACT: A significant increase in the infection level of Baltic cod *Gadus morhua* with the anisakid nematode larvae *Contracaecum osculatum* and *Pseudoterranova decipiens* has been recorded during recent years due to the expanding local population of grey seals *Halichoerus grypus*, which act as final hosts for these parasites. Here, we report from an investigation of 368 cod (total length [TL] 6–49 cm; caught in ICES Subdivision 25) that the infection level of juvenile cod (TL 6–30 cm) with larvae of *C. osculatum* and *P. decipiens* is absent or very low, whereas it increases drastically in larger cod (TL 31–48 cm). A third nematode *Hysterothylacium aduncum* was rarely found. The study indicates that the prey animals for large cod act as transport hosts for the parasite larvae. Analyses of stomach contents of cod caught in the same area (2007–2014) showed that small benthic organisms (including polychaetes *Harmothoë sarsi*) are preferred food items by small cod, the isopod *Saduria entomon* is taken by all size classes, and sprat *Sprattus sprattus* are common prey items for cod larger than 30 cm. Parasitological investigations (microscopic and molecular analyses) of *H. sarsi* (100 specimens) and *S. entomon* (40 specimens) did not reveal infection in these invertebrates, but 11.6% of sprat (265 specimens examined) was shown to be infected with 1–8 *C. osculatum* third stage larvae per fish. Analyses of sprat stomach contents confirmed that copepods and cladocerans are the main food items of sprat. These observations suggest that the *C. osculatum* life cycle in the Baltic Sea includes grey seals as final hosts, sprat as the first transport host and cod as second transport host. It may be speculated that sprat obtain infection by feeding on copepods and/or cladocerans, which could serve as the first intermediate hosts. One cannot exclude the possibility that the size-dependent *C. osculatum* infection of cod may contribute (indirectly or directly) to the differential mortality of larger cod (>38 cm) compared to smaller cod (<30 cm) recently recorded in the Baltic cod population.

KEY WORDS: *Gadus morhua* · *Halichoerus grypus* · *Contracaecum osculatum* · *Pseudoterranova decipiens* · *Hysterothylacium aduncum* · Life cycle · *Sprattus sprattus* · *Saduria* · *Harmothoë*

INTRODUCTION

Baltic cod is a stationary local strain of the Atlantic cod *Gadus morhua* inhabiting the Baltic Sea, a semi-enclosed brackish water area connected to the North Sea through the narrow Danish straits. It is divided

into an eastern and western stock with a mixing zone around the island of Bornholm (Bagge et al. 1994, Hüsey et al. 2016). During recent years a marked increase in the infection level of third-stage larvae of *Contracaecum osculatum* and *Pseudoterranova decipiens* in Baltic cod has been observed

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(Perdiguero-Alonso et al. 2008, Buchmann & Kania 2012, Haarder et al. 2014, Mehrdana et al. 2014, Nadolna & Podolska 2014). This development has been noted during a period with a marked increase of the grey seal *Halichoerus grypus* population (Haarder et al. 2014). These pinnipeds are final hosts for both *C. osculatum* and *P. decipiens* (Marcogliese et al. 1996, McClelland 2002, Mattiucci & Nascetti 2008, Skrzypczak et al. 2014, Lunneryd et al. 2015). Eero et al. (2015) suggested that heavy *C. osculatum* liver infections may partly explain the distress of the eastern population of Baltic cod, where juveniles (<30 cm total length [TL]) perform successfully but larger cod (>38 cm TL) show increased mortality. However, so far no parasitological studies on the infection of juvenile stages of Baltic cod are available, and it is therefore important to address this question. We conducted a basic parasitological investigation of different size classes (TL 6–49 cm) of Baltic cod caught by the research vessel 'DANA' from 2013 to 2015 in order to elucidate any size-dependent infection with larval nematodes. In addition, stomach content analyses of cod from the same size classes were obtained in order to find putative intermediate/transport hosts for the parasite larvae. Samples of main prey organisms identified were then caught during the same research cruises and subsequently examined for presence of parasite larvae.

MATERIALS AND METHODS

Sampling area and fish

A total of 368 Baltic cod *Gadus morhua* (TL 6–49 cm) were examined for presence of parasites (see Table 1). Fish were collected by trawl (water depth 57–77 m; 54.45° to 55.45°N, 014.30° to 016.30°E) from 2013 to 2015 during cruises with RV 'DANA' (National Institute of Aquatic Resources, Technical University of Denmark) except for 11 cod taken by a local fisherman (trawl) at position 55.04°N, 014.50°E. Fish were frozen immediately after catch and kept at –20°C until investigation. We also included examination of 265 specimens of sprat *Sprattus sprattus* (TL 11–13 cm) caught by RV 'DANA' by trawl in the same area and treated as described above. Cod samples from RV 'DANA' were obtained using a TV3 bottom trawl, while sprat samples were obtained by both TV3 bottom trawl and FOTØ pelagic trawl. Samples of *Saduria entomon* and *Harmothoe sarsi*

were caught in the same area with a 1 m dredge and a 2 m beam trawl.

Stomach content analysis

Stomach content recordings from 5363 specimens of Baltic cod (TL 5–50 cm) caught during cruises with RV 'DANA' in ICES Subdivision (SD) 25 in the period 2007–2014 were analysed. Sampling and analysis methodology has previously been described by Huwer et al. (2014). In brief, stomachs were removed from cod, frozen at –20°C on board and transported to the laboratory, where they were thawed, opened, and stomach contents inspected macroscopically. The ingested prey was characterized as (1) *Saduria entomon*, (2) other crustaceans, (3) other benthos, (4) *Harmothoe sarsi*, (5) sprat *Sprattus sprattus*, or (6) herring *Clupea harengus*. The stomach content of 10 specimens of sprat (caught by pelagic trawling in SD25) was spread on microscope slides and examined under the microscope (100–400× magnification) (Leica DM 5000 B).

Parasitological investigation of cod

Following thawing of frozen cod we measured TL (cm) and total body weight (g) for each fish. Each fish was decapitated, internal organs removed and the musculature (fillet part) was removed and examined according to Karl & Leinemann (1993). Briefly, plastic bags of 400 × 200 mm were used for the fillet and then compressed to a layer of around 2 mm thickness using a 12 t hydraulic shop press (AJ Engros). The compressed bags with fish were frozen at –40°C for 24 h before further analysis. The compressed samples were exposed to a 302 nm UV light using MacroVue™ UV-20 Transilluminator (Hoefler®) in a dark room, and any white-bluish fluorescence from worms was observed and marked. Detected nematodes were carefully withdrawn from the muscle tissue for further analysis. Internal organs including cod livers were compressed in other plastic bags as described above, but because *C. osculatum* larvae in liver tissue do not fluoresce under UV-light, the compressed organs were scrutinized under the dissection microscope (8–100× magnification) (Leica MZ125). Nematodes were counted, isolated and identified using light microscopy and PCR with subsequent sequencing according to Mehrdana et al. (2014) as described below.

Parasitological investigation of prey organisms

A total of 100 *Harmothoë sarsi* and 40 *S. entomon* specimens were dissected and examined under the dissection microscope for occurrence of worm larvae. All soft parts were subsequently removed and conserved in 96% ethanol for further processing including lysis, DNA purification and PCR detection of worm-specific DNA. We used the QIAamp DNA stool Mini kit (Cat. No. 515004) for extraction of DNA according to the manufacturer's instructions. A total of 265 specimens of sprat *S. sprattus* were examined for occurrence of worm larvae with the same methods used for cod as described above.

Morphological and molecular nematode identification

All recovered nematodes were examined under the light microscope (Leica DM 5000 B) for genus determination (Mehrdana et al. 2014). Nematodes were subsequently preserved in 96% ethanol (CCS Health Care) for further molecular identification. The middle part of the nematode larva was cut out aseptically and incubated in 100 µl lysis buffer (0.45%, Tween 20, 60 µl ml⁻¹, Proteinase K, 10 mM Tris and 1 mM EDTA) at 55°C, 450 rpm, in the Eppendorf Thermomixer Comfort (Eppendorf AG). Incubation time varied but continued until complete digestion. Proteinase K was then deactivated at 95°C for 10 min, and the lysate was used for PCR amplification. In order to ensure that no worm material was left undetected in *H. sarsi* and *S. entomon*, we also performed PCR on DNA extracted from ethanol conserved soft parts of these invertebrates. PCR was performed in a Biometra T3 thermocycler (Fisher Scientific) using 60 µl reaction volumes. The reaction mixtures consisted of 6 µl lysate as template, 1 unit of BioTaq DNA polymerase (DNA-Technology), 1 mM dNTP, 1.5 mM MgCl₂ and 1 µM of the 2 primers. In order to amplify the internal transcribed spacer (ITS) region, the primers NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') were used as forward and reverse primer, respectively (Zhu et al. 2007). PCR conditions were 2 min of pre-denaturation at 94°C followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and elongation at 72°C for 1 min 15 s. Finally, a post-elongation step was performed at 72°C for 7 min. Products were analysed by 2% ethidium bromide-stained agarose gels. PCR products were purified using Illustra GF PCR

and the Gel Band Purification kit (GE Healthcare, cat. no. 28-9034-71) according to the manufacturer's instructions prior to sequencing at Macrogen Inc. Species identification was based on the sequences encoding the ITS region (18S [3' end], ITS-1, 5.8S rRNA, ITS-2, and 28S rRNA [5' end]).

Statistics and calculations

Stomach content of cod was expressed as the percentage of all stomachs in a certain size class (frequency of occurrence) carrying a certain prey organism. The infection percentages of cod and the mean number of worms per infected fish in the different size classes were calculated. Differences were evaluated by the Mann-Whitney *U*-test. Microsoft Excel 2007 and SigmaPlot 12.5 were used for statistical calculations. A probability level of 5% was used for all analyses.

RESULTS

Parasite infections

Baltic cod with TL below 20 cm showed none or very low infections with nematode larvae. A few fish (TL 21–30 cm) harboured a low infection (1 to 2 parasites per fish) but only with *Contracaecum osculatum* (Table 1, Fig. 1). The occurrence of both *C. osculatum* and *Pseudoterranova decipiens* increased with host size from 31 to 49 cm, particularly with regard to the first species. More than 87% of larger cod harboured *C. osculatum* larvae with a mean number of 16 parasites per fish. A few specimens of a third nematode species *Hysterothylacium aduncum*, using fish as both intermediate and final hosts, were found in fish larger than TL 41 cm. Third stage larvae recovered were identified as *C. osculatum* (92% of worms recovered), *P. decipiens* (5% of worms) and *H. aduncum* (3%) (Table 1). No worm infections were detected in *Harmothoë sarsi* and *Saduria entomon* by any method, but sprat was found infected (liver) with *C. osculatum* (11.6%, 1–8 parasites per fish, mean no. of parasites per infected fish = 1.6) and *H. aduncum* (0.4%, mean no. of parasites per fish = 1.0).

Stomach analyses

Small benthic invertebrates (including *H. sarsi* polychaetes) were preferred by the smallest cod. The

Table 1. Prevalence (P) and mean intensity (I) of nematodes infecting muscle and liver of Baltic cod (various size classes) from ICES Subdivision 25 (Bornholm Basin). Third stage larvae recovered from cod listed as 'All anisakids', *Contracaecum osculatatum*, *Pseudoterranova decipiens*, or *Hysterothylacium aduncum*. *Significantly different from size classes 1–10, 11–20 and 21–30 cm ($p < 0.05$)

| Size class (cm) | No. of fish | Muscle infections | | Liver infections | |
|---|-------------|-------------------|------|------------------|---------|
| | | P (%) | I | P (%) | I |
| All anisakids | | | | | |
| 1–10 | 53 | 0 | 0 | 0 | 0 |
| 11–20 | 194 | 0 | 0 | 2.06 | 1 |
| 21–30 | 21 | 9.52 | 1 | 28.57 | 2 |
| 31–40 | 53 | 3.77 | 1.5 | 75.47 * | 16.1 |
| 41–50 | 47 | 6.38 | 1.67 | 87.23 * | 16.24 * |
| All classes | 368 | 1.9 | 1.43 | 25.82 | 13.96 |
| <i>Contracaecum osculatatum</i> | | | | | |
| 1–10 | 53 | 0 | 0 | 0 | 0 |
| 11–20 | 194 | 0 | 0 | 2.06 | 1 |
| 21–30 | 21 | 9.52 | 1 | 28.57 | 2 |
| 31–40 | 53 | 0 | 0 | 75.47* | 16.1 |
| 41–50 | 47 | 0 | 0 | 87.23* | 16.24* |
| All classes | 368 | 0.27 | 1 | 25.82 | 13.96 |
| <i>Pseudoterranova decipiens</i> | | | | | |
| 1–10 | 53 | 0 | 0 | 0 | 0 |
| 11–20 | 194 | 0 | 0 | 0 | 0 |
| 21–30 | 21 | 0 | 0 | 0 | 0 |
| 31–40 | 53 | 3.77 | 1.5 | 0 | 0 |
| 41–50 | 47 | 4.26 | 2 | 0 | 0 |
| All classes | 368 | 1.09 | 1.75 | 0 | 0 |
| <i>Hysterothylacium aduncum</i> | | | | | |
| 1–10 | 53 | 0 | 0 | 0 | 0 |
| 11–20 | 194 | 0 | 0 | 0 | 0 |
| 21–30 | 21 | 0 | 0 | 0 | 0 |
| 31–40 | 53 | 0 | 0 | 0 | 0 |
| 41–50 | 47 | 2.13 | 1 | 0 | 0 |
| All classes | 368 | 0.27 | 1 | 0 | 0 |

isopod *S. entomon* was found both in small and larger cod. Sprat *Sprattus sprattus* dominated (up to 30%) the stomach content of fish with TL >21 cm (Fig. 1b). Herring *Clupea harengus* was also represented in the largest cod but at a lower rate (<10%). The sprat stomach content was analysed by microscopy which revealed that copepods (*Temora longicornis*, *Centropages hamatus*) and cladocerans *Bosmina* sp. dominated the sprat stomach contents.

Molecular analyses of worms

Molecular analyses of the ITS region of nematode larvae recovered confirmed the identity of the microscopical analysis. In all sequences from *C. osculatatum*, a fragment of 931 nucleotides, excluding the primer binding site, was obtained. The ITS region was se-

quenced from the 3' end of the 18S ribosomal rRNA to the 5' end of the 28S ribosomal rRNA. ITS sequences obtained from *C. osculatatum* recovered from cod and sprat corresponded (GenBank accession numbers for parasites in cod KU306614–306694 and in sprat KU306695–306696) and showed the highest identity with GenBank accession no. AF411203 for *C. osculatatum* found in *Halichoerus grypus* from the Baltic Sea (Bothnian Bay). The sequence similarity was 99.8 to 100% identical to previously recorded GenBank accession numbers. Sequence analyses of corresponding rDNA from *P. decipiens* (KU306721) and *H. aduncum* (KU306719 and KU306720) showed full identity to GenBank sequences from those species isolated from North Sea fish (Atlantic cod and eel-pout, respectively).

DISCUSSION

The present study documented differential nematode infection rates of various size classes of Baltic cod. Small cod remain uninfected or merely lightly infected whereas cod >30 cm TL experience increasing infections especially with *Contracaecum osculatatum* larvae. The life cycle of this anisakid was elucidated in the laboratory by Køie & Fagerholm (1995), who showed that various invertebrates, including copepods, serve as first intermediate hosts. These crustaceans become infected when they feed on nematode larvae released from the parasite eggs delivered by seal. A dominance of adult *C. osculatatum* in Baltic grey seal stomachs was previously documented by Skrzypczak et al. (2014) and Lunneryd et al. (2015), and the increase of fish infections during the last decades may be caused by the rapidly expanding grey seal population (Haarder et al. 2014). Several fish species are able to obtain infection when ingesting the first intermediate hosts, and the present field study documented that more than 10% of the Baltic sprat investigated was infected by up to 8 parasite larvae per fish, which is a higher infection level than previously reported. In 2009 and 2011 only 2 Baltic sprat out of the 187 examined harboured infection (1 larva per fish) (Skrzypczak & Rolbiecki 2015). It may be hypothesized that Baltic cod in SD25 obtain infection with *C. osculatatum* when cod (reaching a TL of 30 cm) physically become able to ingest sprat. Corresponding dynamic associations between cod size, diet and parasites have been recorded previously in Atlantic cod (Münster et al. 2015). It may be speculated that the sprat become infected when feeding on infected copepods and/or cladocerans, although

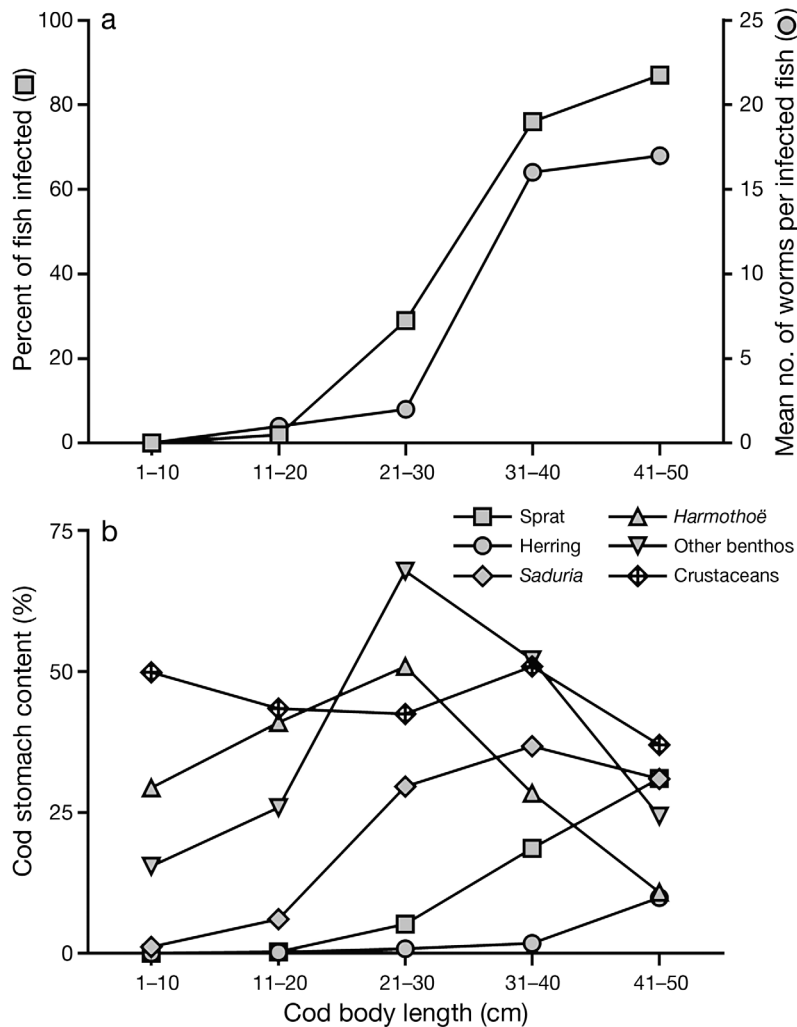


Fig. 1. (a) Percentage of Baltic cod infected with *Contracaecum osculatum* and number of larvae per infected fish in ICES Subdivision 25 (Bornholm Basin) according to size class. (b) Stomach content of cod in the Baltic Sea according to size class. *Saduria*: *Saduria entomon*; *Harmothoë*: *Harmothoë sarsi*

we did not document infection in these planktonic organisms. However, they occur throughout the year all over the Baltic (Ackefors 1969) and are the dominating food elements in sprat (Casini et al. 2004). Baltic herring was included in the Baltic cod diet at a lower rate, but this fish species may also play a role in *C. osculatum* transmission as they may carry, although at a low level, larvae of this nematode (Unger et al. 2014). We were not able, in this study, to detect infection of polychaetes (*Harmothoë sarsi*) and isopods (*Saduria entomon*), but we cannot rule out the possibility that at least part of the infection of cod may be obtained during feeding on these invertebrates. Thus, invertebrate intermediate hosts are generally infected at low prevalences and intensities, and future parasitological studies should include

larger numbers of invertebrates in order to elucidate their potential role as intermediate hosts.

The finding that smaller cod remain uninfected or merely lightly infected until they reach a TL of 30 cm may have implications for the ecology of this fish species in the eastern Baltic Sea. During the last decade, a high survival rate and increasing numbers have been recorded for small cod (TL <30 cm) whereas the population of larger cod (TL >38 cm) has exhibited a drastic and unexpected decline (Eero et al. 2015). Direct predation on cod by seals has been suggested to have caused a reduction in Canadian fish stocks (Chouinard et al. 2005, Swain & Chouinard 2008, Swain & Benoit 2015), and predation may play a role for the Baltic cod as well. However, it may be speculated that parasite infections, mainly due to *C. osculatum*, indirectly affect the performance of the Baltic cod population. Experimental infections of fish with anisakid larvae (*Pseudoterranova* and *Anisakis*) have shown pathological effects in fish tissue (Ramakrishna et al. 1993, Buchmann 2012, Levsen & Berland 2012), depression of host swimming performance (Sprengel & Luchtenberg 1991) and reduced levels of survival (Rohlwing et al. 1998). The pathogenic potential of *Pseudoterranova* and *Contracaecum* larvae is further emphasized by the severe inflammatory reactions induced at the

penetration site when these parasites infect the stomach of mammals including humans (Schaum & Müller 1967, Shamsi & Butcher 2011, Nagasawa 2012, Strøm et al. 2015). Based on these studies it may be hypothesized that the most heavily infected (larger) fish become more vulnerable to predation. The increased and unexpected mortality of larger cod could thereby be associated (directly or indirectly) with an increased level of infection, but this notion should be further investigated by controlled infection studies in the laboratory.

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