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SHORT COMMUNICATION

Infection of North Sea cod, *Gadus morhua* L., larvae with the parasitic nematode *Hysterothylacium aduncum* Rudolphi

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Investigation of 2197 cod larvae and post-larvae collected in the North Sea revealed high prevalence of infection with a parasitic anisakid nematode identified morphologically and genetically as *Hysterothylacium aduncum*. Nematodes were third stage larvae and were almost exclusively found in the body cavity and they were never encapsulated. Prevalence increased significantly from 1992 to 2001 concomitantly with increased sea temperature. The possibility that the extent of parasitism is influenced by temperature change is discussed.

KEYWORDS: cod larvae; *Gadus morhua*; North Sea; climate change; nematode; *Hysterothylacium aduncum*

Studies have shown that fish larvae are susceptible to parasitic nematodes (Rosenthal, 1967; Bristow, 1990; Balbuena *et al*., 2000) and infection is most likely acquired through the ingestion of infected copepods (Køie, 1993). According to laboratory studies, such infections in larval fish can be lethal to both larval herring (Rosenthal, 1967; Balbuena *et al*., 2000) and halibut (Bristow, 1990). In the present study, we investigate the prevalence (number of infected hosts/total number of hosts examined) of parasitic nematodes in larval Atlantic cod (*Gadus morhua* L.) in the North Sea and discuss whether the cod may be negatively affected by infections. The Atlantic cod is one of the most important commercial fish species in north-western Europe, but in the North Sea, the cod stock has declined markedly over the past four decades (Horwood *et al*., 2006). Since 1990, the spawning-stock biomass of North Sea cod has reduced by ~50%, a decline which has been associated with a marked rise North Sea temperature (Horwood *et al*., 2006). The cod population in the North Sea may be particularly sensitive to climatic changes, since this species inhabits coastal areas with yearly mean temperatures close to the upper tolerance limit of cod (Sundby, 2000). We here present results from a survey of nematode infections in cod larvae sampled in 1992 and 2001. This sampling period spans a period during which the mean water temperature in the North Sea has risen and during which the cod stock has declined.

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A total of 2197 cod larvae were collected in the North-eastern part of the North Sea, off Denmark, as previously described (Munk, 1999; Nielsen and Munk, 2004). Samples were collected in April and May at 14 stations (538 fish) and 27 stations (1659 fish) in 1992 and 2001, respectively. Fish larvae were immediately preserved in 96% ethanol and then identified to species. The length of each cod larva was measured and larvae were examined for the presence of parasitic nematodes under a Leica M212.5 stereomicroscope. Nematode larvae were removed from the hosts by dissection and anterior and posterior parts of retrieved parasites, which possessed morphologically important traits such as intestinal caecum, ventricular appendix and excretory pore, were cleared in lactic acid and mounted on slides with glycerol gelatine for morphological examination and identification using a Leica DMLB microscope. Middle parts, approximately one-third of each nematode, were saved for genetic analysis. Nematode DNA was extracted by proteinase K digestion in lysis buffer [Tween 20 (0.45%), proteinase K (60 μL mL^{-1}), 10 mM Tris and 1 mM EDTA] for 1 h at 55°C followed by a 10 min enzyme inactivation at 94°C. The internal transcribed spacer region (containing ITS1, ITS2 and the 5.8S rRNA gene) was PCR amplified in a Biometra T3 Thermocycler using 1 μL of the crude DNA extract as template in 25 μL reaction volumes, 1 unit of Taq polymerase (Bioline no. BIO21040) at 3.0 mM $MgCl_2$ and the primers NC5 and NC2 at 1.0 μM (Zhu *et al.*, 2007). PCR conditions were as described by Zhu *et al.* (Zhu *et al.*, 2007). The ITS region was then sequenced bi-directionally (Macrogen Inc, Rep. of Korea) using the same primers as for the PCR. The 5′ and 3′ ends of the ITS1 and ITS2 sequences were determined by comparing with anisakid ITS sequences obtained from GenBank (Zhu *et al.*, 2007). A total of 35 sequences were obtained from both sampling years and BLAST searches were then performed for corroboration of morphological species determination. Sequences of ITS1, ITS2 and 5.8S from cod larvae and the most similar sequences found through BLAST searches were aligned with ClustalX. Sequence dissimilarities were calculated in MEGA4 based on pairwise analysis (Tamara *et al.*, 2007).

Differences in the prevalence and intensity (number of parasites in a single infected host) of infection between 2001 and 1992 were tested using a generalized linear mixed model (GLMM) approach (Bolker *et al.*, 2009). Sampling in April was less intensive in 1992 than in 2001 and, therefore, only samples collected in May 1992 and 2001 were used in the analysis (a total of 1022 fish, 538 from 1992 and 484 from 2001). This ensured that the fish analysed were comparable in age and size. For prevalence, a binomial error distribution and a logit link function were used, and for intensity a Poisson error distribution and a log link function were used. The dependent response variables prevalence and intensity of infection were modelled as a function of length of larvae (mm) and year of sampling (a two-level factor) as fixed effects and to account for random variation between stations, sampling station (a 55-level factor) was included as a random effect. One additional analysis was made in which fish >55 mm were excluded from the data set (since such large fish were only present in the 2001 samples) and one analysis in which fish were grouped in 5 mm length classes. To test the statistical significance of the fixed year effect, nested models were compared using maximum likelihood estimation and a Laplace approximation (Bolker *et al.*, 2009). The statistical analyses were conducted with R version 2.10.1, using the R-package lme4 (R Development Core Team, 2010).

Total body length of the cod ranged from 5 to 65 mm (Fig. 1) of which 267 (12.2%) were longer than 35 mm and might more correctly be referred to as post-larvae and juveniles rather than larvae. A large number of larvae and post-larvae examined were infected with L3 larval stages of parasitic nematodes. Nematode larvae were 2–21 mm in length, corresponding to approximately 0.3–1.1 mg wet weight, which was up to 3% of the wet weight of the host. Most infected fish hosted only a single nematode (Table I). Only 2.9% of all infected fish hosted more than two nematodes. Mean intensity (average intensity of parasites among the infected hosts) was one or close to one for most sizes of fish larvae (Fig. 2a) and peaked above 1.2 only for size classes in the range between 35 and 59 mm. Mean abundance of nematodes (i.e. number of parasites/total number of hosts) increased with size of larvae, but tended to stabilize at 0.4 parasites per total number of fish above a length of 35 mm (Fig. 2b). Dispersion was always below 1.0 with a peak for the intermediate sized hosts (Fig. 2c). Differences between prevalence and intensity of infection were tested for fish sampled in May 2001 and 1992. The larvae were slightly larger in 2001 (Fig. 1b), but there was no significant interaction effect on prevalence by length of fish larvae and year of sampling (GLMM, $\chi^2 = 0.071$, $P = 0.790$, $df = 1$). Prevalence of parasitic nematodes was significantly higher in 2001 than in 1992 (Fig. 3, GLMM, $\chi^2 = 11.34$, $P = 0.0008$). This was also the case when fish >55 mm were excluded from the data set ($\chi^2 = 12.45$, $P = 0.002$) and when fish were grouped in 5 mm length classes ($\chi^2 = 12.07$, $P = 0.0005$). Parasitic larval nematodes were present in cod larvae down to 10 mm, with prevalence increasing with size of larvae; parasite prevalence in fish larger than 35 mm (post-larvae and

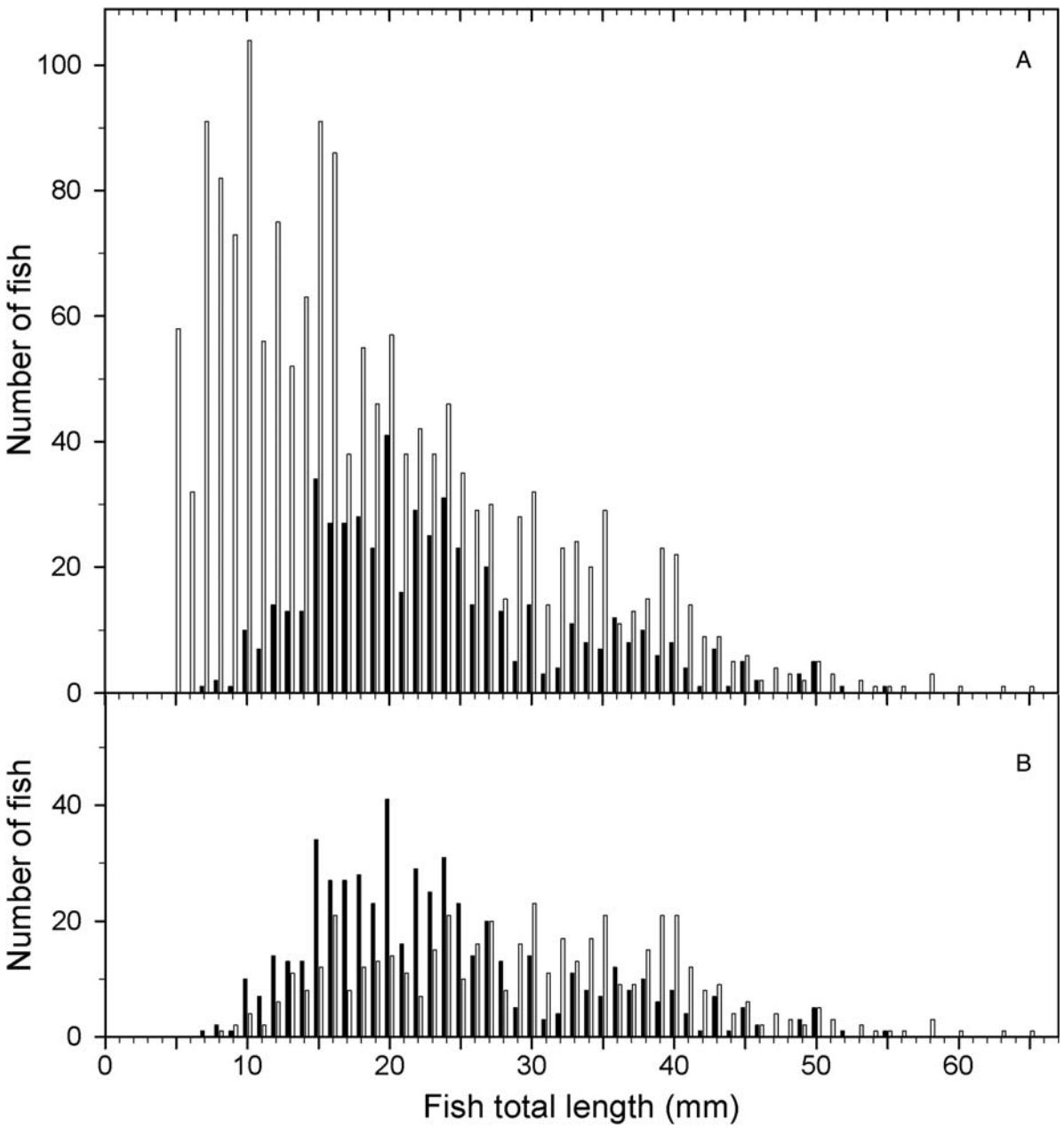

Fig. 1. Length of larval Atlantic cod (*Gadus morhua*) sampled in the North Sea in 1992 ($n = 538$) and 2001 ($n = 1659$). (**A**) All sampled fish; (**B**) exclusively fish sampled in May 1992 ($n = 538$) and May 2001 ($n = 484$). Black bars, 1992; white bars, 2001.

juveniles) reached 35% in 2001. Intensity of infection was significantly higher in 2001 than 1992 (GLMM, $\chi^2 = 12.52$, $P = 0.0004$).

Sequences of the ITS region were obtained from 35 randomly selected nematodes. These included specimens that could be identified as *Hysterothylacium aduncum* based on morphology as well as specimens that could not readily be identified. All 35 sequences were identical (deposited in GenBank under accession number HM598666). BLAST searches showed that ITS1, IT2 and 5.8S sequences of the nematode from North Sea cod larvae were identical to those of *H. aduncum* isolated from fish in the geographically nearby Baltic Sea (Table II). The ITS1 region was 100% identical to *H. aduncum* from the Baltic Sea, whereas ITS1 sequences from *H. aduncum* from various fish species from China and Japan differed at one or two positions (Table II). The ITS2 of *H. aduncum* from North Sea cod larvae was 100% identical to *H. aduncum* and *H. auctum* from the Baltic Sea, and it was also identical to

Table I: Frequency distribution of parasitic nematodes, Hysterothylacium aduncum, in infected North Sea cod larvae sampled in the North Sea in 1992 and 2001

Number of nematodes	Number of hosts	% hosts
1	143	84.1
2	22	12.9
3	3	1.8
4	2	1.2

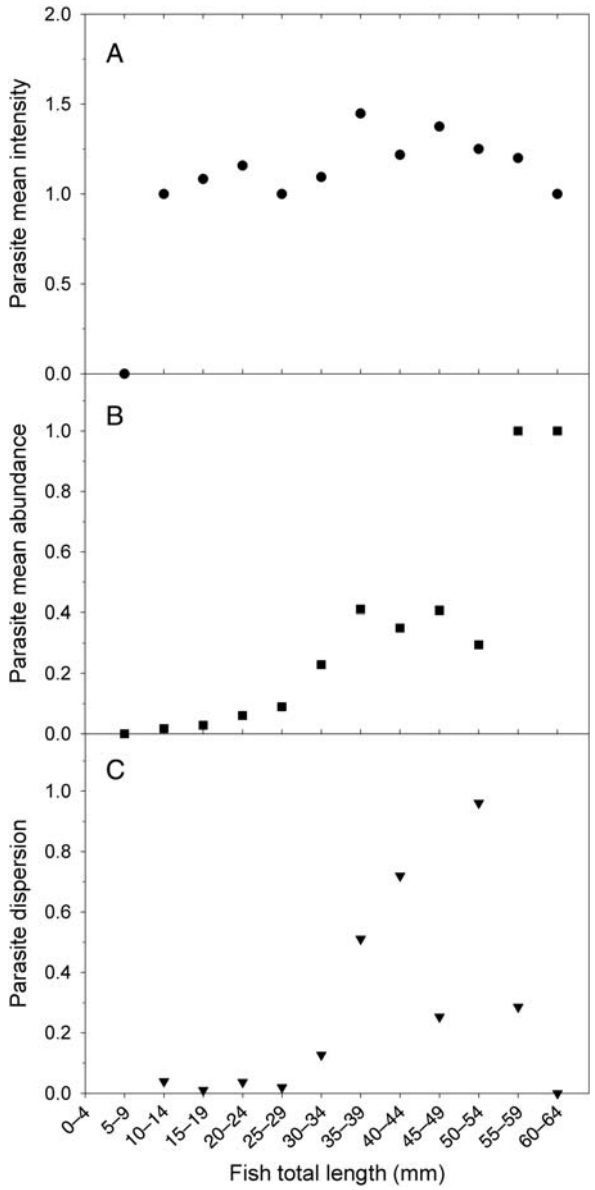

Fig. 2. Infection of larval North Sea cod (*Gadus morhua*) with the parasitic nematode *Hysterothylacium aduncum* in relation to size group of fish. (**A**) Mean intensity (average number of parasites among the infected hosts). (**B**) Mean abundance (number of parasites/total number of hosts). (**C**) Dispersion expressed as average variance to mean ratios of abundance.

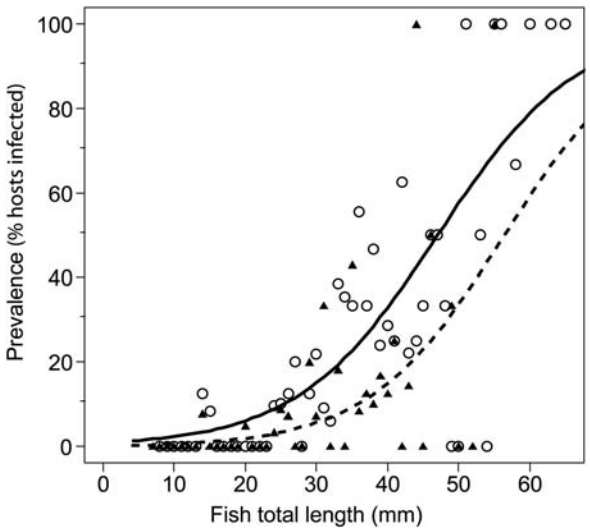

Fig. 3. Prevalence of the parasitic nematode *Hysterothylacium aduncum* in larval North Sea cod (*Gadus morhua*) in May 1992 (black triangles) and May 2001 (white circles). Points represent fractions of infected fish larvae of similar length. Lines represent logistic regression lines for 1992 (dashed line, null deviance = 72, residual deviance = 43) and 2001 (solid line, null deviance = 133, residual deviance = 60).

Table II: Comparison of ITS1, 5.8S and ITS2 sequences from parasitic anisakid nematodes

Species GenBank accession numbers	ITS1	5.8S	ITS2
H. aduncum, North Sea HM598666 (present study)	–	–	–
H. aduncum, Baltic Sea AJ937672–73, AJ225068–70	0	0	0
H. aduncum, China/Japan GQ118683–90, AB277826	1–2	0	0[a]
H. auctum AF115571	4	1	0
H. bidentatum AY603539	20	1	19
Species from other genera, including *Contracaecum* sp. FJ009683	18 ≤	0 ≤	21 ≤

Number of nucleotide differences between sequences from *Hysterothylacium aduncum* from North Sea cod larvae and most similar sequences found by BLAST searches. Sequences were aligned and dissimilarities were calculated in MEGA4 based on pairwise analysis (Tamara *et al.*, 2007). All positions containing gaps and missing data were eliminated from the data set. Numbers in left column represent GenBank accession numbers.
[a]TG insert at position 78–79.

sequences of *H. aduncum* from China and Japan except for a TG insert at position 78–79 in the Asian sequences. The 5.8S rRNA gene was more conserved among nematode species. For this gene, all sequences from *H. aduncum* were identical regardless of sampling location (Table II). This low genetic variability of *H. aduncum* ITS1 and 5.8S rRNA genes is consistent with a previous study concluding that no cryptic speciation existed in *H. aduncum* (Klimpel *et al.*, 2007).

The life cycle of *H. aduncum* includes a host alternation in which marine invertebrates fill in the role of intermediate and/or transport hosts and piscivorous fish represent the final hosts (Køie, 1993). This life cycle has been demonstrated through laboratory experiments in which copepods served as first intermediate host (Køie, 1993), and *H. aduncum* larvae have been found in several species of copepods and other invertebrates (Marcogliese, 1996). The cod larvae must have become infected with nematode larvae upon ingestion of infected copepods. Hence, even though Atlantic cod is the final host of *H. aduncum*, cod larvae are able to fill in the same role as second intermediate/transport hosts, i.e. transport of third stage *H. aduncum* larvae to a final host. *Hysterothylacium aduncum* larvae can infect cultured larvae of Atlantic cod (Karlsbakk *et al.*, 2001), but the present report is the first documentation of *H. aduncum* in wild North Sea cod larvae. It is possible that *H. aduncum* exerts an important biological pressure on larval North Sea cod. Most larvae were infected with only one nematode (Table I, Fig. 2a). This suggests that infection intensity higher than one may be lethal to the smallest larvae. Parasite-induced host mortality tends to lead to peaked, or bell-shaped, age-abundance curves, whereas the degree of dispersion will decline in the older age classes (Anderson and Gordon, 1982). We did observe some, albeit weak, tendency for both these phenomena (Fig. 2b and c).

Hysterothylacium aduncum is considered non-pathogenic or slightly pathogenic in larger fish and it is common to find numerous nematodes in a single adult host (Hamre and Karlsbakk, 2002). Larval fish, on the other hand, seem to be more severely affected by nematode infection. When a nematode larva enters the digestive tract of a fish larva, it will penetrate the wall of the stomach or the intestine and move around in the visceral cavity of the host. This movement of a parasitic nematode has shown to be lethal to herring and halibut larvae up to at least 25 mm in length (Rosenthal, 1967; Bristow, 1990; Balbuena *et al*,. 2000) and such an effect is also be expected for the smallest cod larvae shown to be infected with *H. aduncum* in the present study. Indeed, Karlsbakk *et al.* (Karlsbakk *et al.*, 2001) found morphological changes of the belly of 25–33 mm long cod larvae that had died following infection with *H. aduncum* larvae.

Hamre and Karlsbakk (Hamre and Karlsbakk, 2002) reported high prevalence of *H. aduncum* larvae in juvenile Mueller's pearlside, *Maurolicus muelleri*. Adults of this small fish reach a maximum of 7–8 cm and 1-year juveniles, which are close to sexual maturity, are thus not much larger than many marine fish larvae. The prevalence of 67–98% and the fact that nematodes were found encapsulated in the viscera in *M. muelleri* (Hamre and Karlsbakk, 2002) suggest that the nematodes were pacified by the host's immune defence and that infection was not lethal. In the present study, the *H. aduncum* larvae found in cod larvae were almost exclusively found outside the gut and were never encapsulated and, thereby, capable of moving around inside the host. This suggests that the immune response of the cod larvae was not able to counteract the intruding nematode. The immune system is not fully competent in cod larvae until they have exceeded at least 33 mm (Schrøder *et al.*, 1998), and this lack of an adequate immunological response in cod larvae and post-larvae leads to them being more severely vulnerable to parasitic nematodes than larger and juvenile fish.

Hysterothylacium aduncum is common in the entire Northern hemisphere (Køie, 1993). Optimum temperature for development of *H. aduncum* is 13°C (Iglesias *et al.*, 2002) and the species is also found in waters that are considerably warmer than the North Sea (Rello *et al.*, 2008). Spawning of North Sea cod takes place in late winter and early spring (Brander, 1994) when the water temperature has its seasonal minimum, generally between 4 and 8°C (Hughes *et al.*, 2010). Cod larvae and post-larvae, therefore, typically experience temperatures lower than the optimum temperature for *H. aduncum*. This implies that the parasite's metabolism and growth will not be negatively affected by the current temperature increase in the North Sea. On the contrary, it is possible that the parasite will benefit from moderately elevated temperatures. Climate change is thought to have a negative effect on the cod stock in the North Sea (O'Brien *et al.*, 2000). However, the negative effect of climatic change is not limited to the direct effect of temperature increase on cod physiology alone; we hypothesize that climatic changes may also reinforce growth of parasites that are less sensitive to elevated temperature. This implies that the impact of parasitism on North Sea cod larvae might be amplified in the future if water temperature continues to increase.

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