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Comparative biology of common and grey seals along the Dutch coast : stranding, disease, rehabilitation and conservation

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Chapter

7

Hooknose *Agonus cataphractus* and juvenile flatfish as potential intermediate hosts of seal lungworm *Parafilaroides gymnurus*

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Abstract

Verminous pneumonia caused by the lungworms *Otostrongylus circumlitus* and *Paraflaroides gymnuris* is currently the primary cause of disease in common seals *Phoca vitulina* of the Dutch Wadden Sea. Juvenile seals become infected after weaning when they feed on fish that harbour larvae of these parasites. We investigated which Wadden Sea fish species carry lungworm larvae and may thus serve as intermediate hosts. A wide range of fish species were included that were considered to be part of the diet of weaned common seals, such as young flatfish, small-bodied fish species, and shrimp. No lungworm larvae were found in shrimp, despite shrimp being reported as the main diet of seals after weaning. First-stage *P. gymnuris* larvae were found in hooknose (*Agonus cataphractus*), juvenile European plaice (*Pleuronectes platessa*) and in juvenile common dab (*Limanda limanda*). This is the first record of *P. gymnuris* in hooknose. Lungworm larvae were previously found in European plaice and common dab, however our study shows that also juvenile specimens of these flatfishes carry lungworm larvae. Experimental infections, similar to those performed on flatfish that have been reported in the literature, are required for hooknose to test whether the larvae develop to the infective stage in this species. No *O. circumlitus* larvae were detected in any of the fish collected in this study.

Introduction

Parasitic bronchopneumonia is currently the primary cause of disease in common seals *Phoca vitulina* of the Dutch Wadden Sea. In contrast, the other Wadden Sea seal species, the grey seal, *Halichoerus grypus*, rarely suffers from parasitic bronchopneumonia. This disease can be caused by two lungworm species of the superfamily Metastrongyloidea; *Otostrongylus circumlitus* (Railliet 1899) of the family Crenosomatidae and *Parafilaroides gymnurinus* (Railliet 1899) of the family Filaroididae (Dailey 2006). Common seals can be infected with either one of these species, or they can have a mixed infection (Claussen *et al.* 1991; SRRC unpublished data). Although Gosselin and Measures (1997) described *P. gymnurinus* from Canadian grey seals, live-stranded grey seals on the Dutch coast were found to have parasitic pneumonia with solely *O. circumlitus* infections (SRRC unpublished data). There has been a sharp increase in the number of juvenile stranded common seals with severe parasitic pneumonia since the late 1990s (see Chapter 1, Appendix 1). Currently, the number of stranded juvenile seals constitutes about a third of the roughly 1500 pups that are born annually in Dutch waters (aerial survey data; Strucker *et al.* 2013; TSEG 2013).

It is generally believed that the transmission of seal lungworms is horizontal via the food chain, with seals becoming infected after weaning when they feed on fish that harbour larvae of these parasites (Measures 2001). The large seal lungworm, *Otostrongylus circumlitus*, is usually found in the bronchi and bronchioles and occasionally in the heart and pulmonary arteries of seals, whereas the small lungworm, *Parafilaroides* spp., are usually embedded in the respiratory parenchyma (Elson-Riggins 2001; Measures 2001). Mature lungworms are ovoviparous and release first-stage larvae (L1). These larvae can be coughed up or moved passively up in the airways, after which they are swallowed and passed with the faeces to the external environment. When L1 are ingested by an intermediate host or possibly after ingestion of a paratenic host (transport host in which no larval development occurs), these L1 will moult into second-stage larvae (L2) and subsequently into third-stage larvae (L3) which is the stage that is infective to seals (Measures 2001).

There are two studies in the literature examining the lifecycle of seal lungworms; both suggest that flatfish serve as intermediate hosts. The experimental work of Bergeron *et al.* (1997) suggests that in Canadian waters American plaice (*Hippoglossoides platessoides*) may serve as an intermediate host for *O. circumlitus*. Experimental infections also suggested that turbot (*Psetta maxima*) may be an intermediate host for *O. circumlitus* (Lehnert *et al.* 2010). In these two studies, L3 were recovered from the gastrointestinal tract of the flatfishes, after experimentally infecting them with live first-stage *O. circumlitus* larvae. Lehnert *et al.* (2010) also examined the presence of lungworm larvae in naturally infected fish caught in German waters. They found ensheathed larvae (L2 or L3) of *P. gymnurinus* in European plaice (*Pleuronectes platessa*) and common dab (*Limanda limanda*) and suggest that these species serve as intermediate hosts. The lengths

of the examined fishes were not given by Lehnert *et al.* and it is unknown whether they detected larvae in juvenile flatfish.

The only other intermediate host identified thus far for the genus *Parafilaroides* is a small tidepool fish, *Girella nigricans*. This coprophagous fish serves as an intermediate host for *P. decorus* off the coast of California, which parasitises the California sea lion (*Zalophus californianus*) (Dailey 1970).

In recent years, common seals were found to suffer from parasitic bronchopneumonia at a much younger age than found in previous decades (Chapter 1 and 2). Currently, the strandings of the first moribund lungworm cases are in August, when seals are one to three months of age. These first cases of stranded seals were found to suffer from *O. circumlitus* rather than *P. gymnuris* infection (SRRC unpublished data). The discovery of extremely young common seals infected with mature *O. circumlitus* is of interest because it suggests that they are infected when first feeding in the tidal pools or even prior to weaning. Thus, the diet of weaned seals must contain fish or invertebrates species that carry lungworm larvae or alternatively, vertical transmission - that is from mother to pup - may play a role (Chapter 8). It is unlikely that adult specimens of flatfish species such as turbot (Lehnert *et al.* 2010) infect young seals as they are too large to be eaten. The most suitable fish species to use as an intermediate host would be small-bodied enough to serve as prey, i.e. they should be small-bodied fish species or juvenile specimens of larger species.

Havinga (1933) conducted a diet study of shot seals in the Netherlands in the 1930s and reported that after weaning, young seals exclusively feed on shrimp for six weeks. Thereafter, juvenile and subadult seals (categorised by Havinga as seals up to a body length of 150 cm) mainly forage on herring (*Clupea harengus*), flounder (*Pleuronectus flesus*), European plaice (*Pleuronectus platessa*), eelpout (*Zoarces viviparus*) and a mixed category (due to otolith similarity) of shorthorn sculpin (*Myoxocephalus Scorpius*) and hooknose (*Agonus catapraetus*). Havinga's findings are in line with those of Sergeant (1951) who conducted a diet study of shot seals from The Wash (UK) in the 1940s. Sergeant also reported a transitional shrimp-eating stage after weaning, followed by a mixed diet in the first winter consisting of fish, crabs, as well as shrimp.

It is unknown whether pups incidentally forage during the four week lactation period. Van Haaften (1974) suggested that seals incidentally catch fish during this period. However, both Sergeant (1951) and Havinga (1933) found that pups were feeding exclusively on milk for at least a month after birth.

We hypothesised that weaned seals become infected with lungworms via small-bodied fishes that are part of their diet. Therefore, we studied the presence of lungworm larvae in a range of fish species that are considered to be part of the diet of young common seals. In addition, we explored the possible causes for the high disease rate of lungworms in common seals.

Materials and Methods

Fish catch

Multiple catches of fish and shrimp were retrieved from a commercial shrimp fisherman. The fish catches were made in different months of the year and were all from a location within a few kilometres distance of the shoreline of the Wadden Sea islands: 1. February 14th 2013, north of Ameland, 2. April 18th 2013, north of Schiermonnikoog, 3. July 18th and 31st 2013, north of Schiermonnikoog. On July 15th 2013 an additional sample of fish was caught 2 km west of the harbour of Lauwersoog (mainland provinces Friesland/Groningen). The catches included a wide range of pelagic and demersal fish and invertebrates. Species were identified using Moen and Svenson (2004) and Zoetemeyer *et al.* (2009). Fish species that were included in the three sessions (February/April/July) were: European plaice (*Pleuronectes platessa*), common dab (*Limanda limanda*), flounder (*Platichthys flesus*), sole (*Solea solea*), brill (*Scophthalmus rhombus*), goby (*Pomatoschistus* spp.), sprat (*Sprattus sprattus*), smelt (*Osmerus eperlanus*), greater sandeel (*Hyperoplus lanceolatus*), herring (*Clupea harengus*), whiting (*Merlangius merlangus*), Atlantic horse mackerel (*Trachurus trachurus*), three-spined stickleback (*Gasterosteus aculeatus*), hooknose (*Agonus cataphractus*), fourbeared rockling (*Enchelyopus cimbrius*), gunnel (*Pholis gunnellus*), shorthorn sculpin (*Myoxophalus scorpius*), dragonet (*Callionymus lyra*), and river lamprey (*Lampetra fluviatilis*), viviparous blenny (*Zoarcas viviparus*). The invertebrates examined were shrimp (*Crangon crangon*), polychaetes and crab (several species that occur in the Wadden Sea). All fish and invertebrate samples were photographed and measured.

Preparing fish samples

Each sample included one or several specimens of a fish species. For the February catch, whole fishes were digested whilst in April and July the gastrointestinal tracts were digested separately from the remainder of the bodies. We focused on the gastrointestinal tract since fish experimentally infected with pinniped lungworm larvae had larvae in the intestinal mucosa, the circular muscle layer of the intestine, the intestinal serosa and mesenteric adipose tissue (Dailey 1970; Bergeron *et al.* 1997; Lehnert *et al.* 2010). The maximum weight per sample was 40 gram.

Tissue digestion

The isolation of larvae from fish was conducted by tissue digestion, according to the method of Patel *et al.* (2014) with the following modifications. Each species was digested separately from other species, but if more than one individual of a species was represented in the catch then several individuals were digested together, in batches of up to 1 L of tissue/digestion fluid. The fish tissue was cut into small pieces and incubated in artificial digestion fluid (0.7% pepsin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 0.5% hydrochloric acid) at a ratio of 25 ml per gram of tissue. Incubation was performed in a stirring water bath at 37 °C for 1-3 hours or until all tissue was digested. If the solution

included non-digested material such as shrimp carapaces, then the digest was sieved (sieve aperture: 1.5 - 3.3 mm). The samples were either centrifuged (200 rpm for 4 minutes) or left overnight to sediment. The supernatant was removed by pipetting and replaced by phosphate buffered saline (PBS). Samples were stored on ice until examination. If it was not possible to examine the digests within a week, they were frozen at -20 °C and examined after thawing.

Microscopy

The digests were screened using a microscope (Olympus SZ51 Zoom stereomicroscope). For the February and April catches, random samples were taken from the digest, i.e. not all digestion fluid was checked. For the July samples, all of the digest from 10 gram of gastrointestinal tract was checked. Larvae were photographed with a compound microscope (Biostar and Basic Biological microscopes, Exacta+Optech GmbH, Munich, Germany) equipped with a camera (Moticam 1000, Motic Deutschland GmbH, Wetzlar, Germany). A few of the February digests were shipped on ice to the Royal Veterinary College, UK, and examined on an Olympus SZ51 Zoom stereo microscope and a Laborlux 11 compound microscope (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK) and photographed using a Bresser LCD-Micro compound microscope (Meade Instruments Europe GmbH and Co. KG, Rhede, Germany).

Molecular analyses

DNA from larval nematodes was extracted using a QIAamp DNA Micro Kit (QIAGEN Benelux B.V, Venlo, the Netherlands and Crawley, UK). The manufacturer's procedure was followed, with the following adaptation: larvae were either stored at 4 °C or frozen after the proteinase K incubation step.

PCR reactions were performed using two different methods, both utilizing a 55 °C annealing temperature. Both methods used NCA primers, which were based on the sequence of the NC primers designed by Gasser *et al.* (1993). The NC primers amplify the ITS-2 region of rhabditid nematodes. However, they do not optimally amplify the ITS-2 region of European *O. circumlitus* (personal observation, N. Osinga and J. Elson-Riggins). This is due to sequence mismatches of the primers to European *O. circumlitus*. We changed one base in the NC1 primer to make primer NC1A and we designed a completely new reverse primer that amplified 527 bp for *O. circumlitus* and 575 bp for *P. gymnuris* from European seals. The NCA primer sequences were NC1A: 5'-ACATCTGGTTCAGGGTTGTT-3' and NC2A: 5'-GTTTCAGCGGGTAATCACATC-3'. These primers were optimized using DNA from adult *P. gymnuris* and *O. circumlitus* obtained from seals at necropsy.

The first PCR method used 10 µl reaction volumes and a KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Inc., Wilmington, MA, USA), according to the enzyme manufacturer's instructions. Forward primers were 5'end labelled with a 6-HEX fluorescent dye. An Eppendorf Mastercycler (Eppendorf, Nijmegen, the Netherlands) was used and amplified fragments were sized on an ABI Prism 3130 Genetic Analyzer

using GeneScan 500 ROX size standard (Applied Biosystems, Bleiswijk, the Netherlands). The second method used 25 µl reaction volumes and a MyTaq HS DNA Polymerase kit (Bioline, London, UK), according to the manufacturer's instructions, and unlabelled primers in a G-storm GS1 thermal cycler (GRI, Brain tree, UK). Products were visualized on 1.5% agarose gels stained with Safe view (NBS biological Ltd., Huntington, UK). In all experiments, a positive and negative control (no DNA) was included.

PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and sent for sequencing to either BaseClear (Leiden, the Netherlands) or GATC (Constance, Germany). The sequence results were BLASTed against the GenBank database.

Results

Shrimp were abundant in the fisherman's catches and many specimens were included in all three digestion sessions. However, no nematodes were detected in any of the shrimp investigated.

Eighteen nematode larvae were collected from digests of hooknose. Four of these 18 morphologically were clearly not lungworm larvae, and the DNA from these larvae did not generate a product with the NCA primers. Of the remaining 14 larvae (February n=7, April n=4, July n=3), successful sequences were obtained from 9 larvae. These all BLASTed to *P. gymnurus*. No *O. circumlitus* larvae were detected in hooknose. The average larval length of the 9 sequenced larvae was 299 µm (± 30 µm), with lengths ranging between 255 µm and 340 µm (Figure 1). No sheaths were observed on any of the 14 larvae.

Lungworm larvae were also found in European plaice and common dab. From European plaice, a total of 22 larvae were collected. Two of these 22 morphologically were clearly not lungworm larvae, and the DNA from these larvae did not generate a product with the NCA primers. Of the remaining 20 larvae (April n=17, July n=3), successful sequences were obtained from 7 preparations of larvae, these all BLASTed to *P. gymnurus*. One of these positive sequences was a sample that included 6 of the 20 larvae together in one tube because of similarity in size and morphology. No *O. circumlitus* larvae were detected in European plaice. Since we do not know which larva of the mixed sample was positive; the measurements of these 6 larvae were excluded. The average larval length of the remaining 6 single sequenced larvae was 313 µm (± 21 µm), with lengths ranging between 323 and 330 µm. No sheaths were observed on any of the six larvae investigated individually or in any the six larvae investigated together. However, among the larvae with negative pcr results, one ensheathed larva of 690 µm was found (Figure 2) and one partial larva was found that was also ensheathed. The morphology of the tail of the entire ensheathed larva was consistent with that of the L1 larvae that BLASTed to *P. gymnurus*.

From common dab, a total of 30 larvae (February n=29, April n=1) were collected. Successful sequences were obtained from 14 larvae. Of these 14 larvae, 5 BLASTed to *P. gymnurus*, The other 9 larvae BLASTed to *Pseudalius inflexus*, which is a lungworm of harbour porpoises (*Phocoena phocoena*) (Carreno & Nadler 2003). No *O. circumlitus* larvae

were detected in common dab. Measurements were available for 3 of the 5 *P. gymnurus* larvae. The average larval length of these 3 larvae was 338 μm ($\pm 28 \mu\text{m}$), with lengths ranging between 345 and 385 μm . No sheaths were observed on any of the 14 larvae.

The *P. gymnurus* larvae were found in hooknose from catches representing all the sampling months; February 2103 (Ameland), 2. April 2013 (Schiermonnikoog), and 3. July 2013 (Schiermonnikoog). The *P. gymnurus* larvae from European plaice were found in the catches of April and July, and those from common dab were found in the catches of February and April. The hooknose specimens from these samples measured on average 7.7 cm (± 1.3 cm, range 5.4-10.3 cm, $n=55$). The length at maturity is not described for the hooknose, only a common length of 14 cm and a maximum length of 21 cm has been



Figure 1. Nematode larva (L1), collected from hooknose digest in April 2013 (length 350 μm), bar=100 μm .

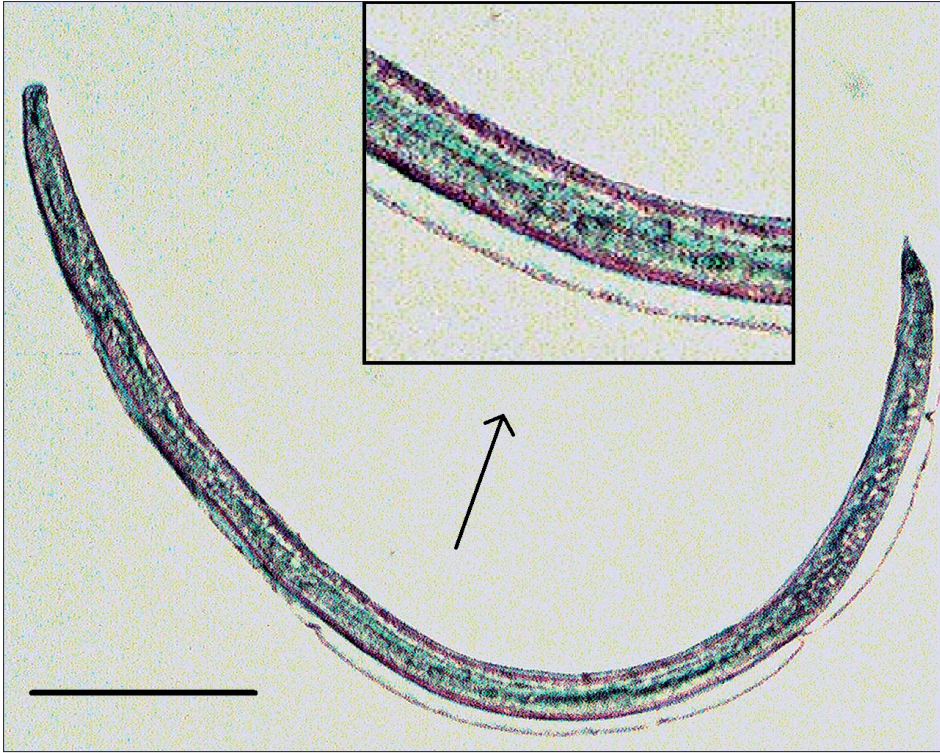


Figure 2. Ensheathed nematode larva, collected from European plaice digest in July 2013 (690 μm), bar=100 μm . Inset shows larval sheath.

reported for this species (Froese & Pauly 2014). It therefore appears that our specimens were not full grown. The average length of European plaice was 6.8 cm ($\pm$ 1.5 cm, range 6.1-7.7 cm, $n=21$) and the average length of common dab was 9.2 cm ($\pm$ 0.4 cm, range 8.8-9.5 cm, $n=3$). These are all classified as juvenile fishes; both European plaice and common dab smaller than 10 cm are categorized as 0-group fish (<1 year) (Kuipers 1977; Bolle *et al.* 1994). Seal lungworms were not detected in any of the other fish species or invertebrates that were included in the study.

Discussion

This is the first description of *P. gymnurus* larvae from hooknose (also called armed bullhead or Pogge), *Agonus cataphractus* (Linnaeus, 1758). The finding of *P. gymnurus* larvae in wild European plaice and common dab in the current study is in agreement with the results of Lehnert *et al.* (2010), who also found larvae of this lungworm in these flatfish species. Since previous studies have not distinguished between juvenile and adult flatfishes, this is the first study which shows that juvenile flatfish carry seal lungworm larvae.

Method

The tissue digestion method enabled a high throughput of samples. However, since we digested all gastrointestinal tissue, we were unable to distinguish whether the larvae were actually embedded in the intestinal tissue or were part of the gastrointestinal content i.e. in the prey species of the fish.

It is very difficult to identify larvae using morphology alone (Moritz & Hillis 1996) and the small size of the larvae also made the molecular analyses challenging. Many of the larvae we collected appeared to be *Metastrongyloids*, but the PCR results produced faint bands and/or small peaks, which we were unable to sequence. Also, some of our PCR products yielded poor quality sequence results. Therefore, the number of *P. gymnur* larvae we report here is expected to be an underestimate of the true number of larvae present in our samples. Although ensheathed larvae were found in European plaice, we were unable to successfully amplify these larvae. This may be because the larvae were a species that did not amplify with our primers or because of difficulties with the DNA extraction. For the complete ensheathed larva, the presence of multiple cuticle layers may have prevented lysis of the larva. This may have occurred because we did not include a mechanical homogenization step in the DNA extraction procedure.

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Larval stage of larvae collected

All the successfully sequenced *P. gymnur* larvae collected from hooknose were less than 340 µm in length. According to Gosselin and Measures (1997) *P. gymnur* L1 of Canadian seals are 220-304 µm long. The length of our hooknose larvae, and the fact that they were not sheathed, suggests that they were the L1 stage. Fish can be either intermediate hosts which carry infective larvae or they can be paratenic (transport) hosts. Since we did not encounter third stage larvae, we have no definite proof of hooknose being an intermediate host species. However, the high rate of infection with L1 larvae indicates that hooknose may be an important part of the lifecycle. Future studies involving the experimental infection of hooknose with *P. gymnur* L1 are required to determine whether these larvae successfully moult into the infective third stage.

Species in which larvae were detected

Shrimp is often recorded as the first diet of weaned seals (Havinga 1933; Sergeant 1951). No lungworm larvae were however found in shrimp in the current study and therefore shrimp does not appear to be part of the seal lungworm life cycle in the Wadden Sea. These diet studies suggest that shrimp is the sole source of nutrition for newly weaned common seals. Our results suggest otherwise, therefore studies on the diet of newly weaned seals are needed to better understand the feeding ecology of newly weaned common seals in the Wadden Sea.

The hooknose is a small fish which occurs mostly over soft substrates of sand, mud and gravel along coastal areas. The species is short lived (3–4 years) and has a dietary preference for juvenile crabs (*Carcinus maenas*), shrimp (*Crangon crangon*) and amphipods

(*Gammarus* spp.) (Power & Attrill 2002). The hooknose is only present in subtidal waters and they do not migrate up the whole intertidal zone during high tide (Amara & Paul 2003). Seasonal distribution patterns of hooknose have not been described for the Wadden Sea (Greenwood & Hill 2003), however high winter/low summer abundance was found for the Delta area in the southwestern Netherlands (Hostens 2000) and the estuaries of the UK (Power & Attrill 2002; Greenwood & Hill 2003). Two monitoring studies of hooknose in Dutch waters are available. The first reports that hooknose abundance in the North Sea for the period 1990-1995 was higher than found in the beginning of the 20th century (Rijnsdorp *et al.* 1996). The second study reports a decreasing trend for the period 1970-2006 for three Dutch coastal regions; the Dutch Wadden Sea, the Westerschelde and the Dutch coastal zone (Tulp *et al.* 2008). Previous studies investigating the parasites of hooknose found larvae of the sealworm *Pseudoterranova decipiens* (Andersen *et al.* 1995) and four other nematode species (*Hysterothylacium aduncum* (L3/L4 stage and adult stage) and *Ascarophis arctica*, *Capillaria gracilis*, *Cucullianus heterochrous* (all in adult stage) (Klimpel *et al.* 2003).

The hooknose was found in the stomachs of both young and adult common seals in Dutch waters in the 1930s (Havinga 1933). Hooknose was also reported to be a part of the common seal diet in Schleswig-Holstein, Germany (Behrends 1985). Also, hooknose was reported to be part of the diet of common seals in Scotland, but with a low occurrence (Tollit & Thompson 1996; Sharples *et al.* 2009).

The finding of *P. gymnurus* lungworm larvae in wild European plaice and common dab in the current study is in agreement with the results of Lehnert *et al.* (2010), who found ensheathed (L2 or L3) *P. gymnurus* larvae in these fishes. Also, in an experimental setting, turbot was suggested to be an intermediate host for *O. circumlitus* (Lehnert *et al.* 2010). The finding of lungworm larvae in European plaice, common dab and turbot, combined with earlier findings of seal lungworms in American plaice from Canadian waters, suggests that a wide range of flatfish species can serve as intermediate hosts for seal lungworms.

Flatfishes were reported to be an important component of the diet of both juvenile and adult common seals in Dutch waters (Havinga 1933). In the study of Havinga, flounder (*Platichthys flesus*) was reported as the most abundant flatfish species in seal stomachs, but also European plaice and common dab were frequently found. For European plaice, Havinga distinguished fish length categories; plaice with estimated sizes less than 10 cm (which are juveniles) were consumed two times more frequently than plaice with estimated sizes of more than 10 cm.

Although larvae were found in pelagic fish species, none of these larvae appeared morphologically to be seal lungworm larvae and also no positive PCR results were obtained from any of these larvae. This finding suggests that pelagic fish species are not part of the seal lungworm lifecycles. The distribution of fish in the water column is expected to affect its importance as an intermediate host. Because demersal fish species occur in the lower

water column, they are expected to be infected with a higher number of L1s that have sunk to the seabed after defecation by seals.

Kontramavichus and Delyamure (1979) suggested, based on experimental exposure, that polychaetes serve as intermediate hosts of *P. gymnurinus*. However the larvae found in the exposed polychaetes were of a length suggestive only for first larval stage (392 µm). A number of polychaetes were included in this study and digests were randomly checked. However, no larvae were detected in the current study.

Interestingly, *O. circumlitus* larvae were not detected in the current study or in the study of Lehnert *et al.* (2010). This is despite primers being used that could amplify DNA of both lungworm species. Future studies should continue to focus on the search for the intermediate hosts of *O. circumlitus*.

Route of infection to seals

Based on the findings of the current study, juvenile seals most likely become infected with *P. gymnurinus* larvae when they prey on hooknose and/or juvenile flatfish after weaning. These fish species are abundant in Dutch coastal waters and they were also abundant in our catches. We found that flatfishes that are younger than one year carry ensheathed larvae. Possibly, there are differences in larval load between juvenile and adult fish and this should be investigated in future studies. In cod (*Gadus morhua*) the number of sealworm (*Pseudoterranova decipiens*) larvae per fish increased with the length and age of the fish (Andersen *et al.* 1995). In contrast, it could also be that the larval load is especially high in juvenile flatfish as they have a more coastal distribution (van Beek *et al.* 1989) and may therefore be exposed to a higher number of defecating seals. In a study of the prevalence of sealworm *Pseudoterranova decipiens* in Norwegian cod, Aspholm *et al.* (1995) found that the larval load in cod decreased with increasing distance to the seal haul out areas. The recent population increase of both common and grey seals (CBS *et al.* 2013) in Dutch waters may have increased the number of nematode larvae present in coastal waters.

Experimental infection of flatfish with of *O. circumlitus* has shown that in fish, L1 moult to L3 within two weeks (Lehnert *et al.* 2010). In May, adult common seals migrate to coastal waters to give birth. During the four week pupping season, there is a high concentration of defecating seals and there is sufficient time for larvae to mature during this period. This may lead to an increased load of infective larvae in coastal fish at the end of the pupping season which subsequently infects weaned seals that forage near the pupping sites.

High disease rate in common seals

Contrary to common seals, the other North Sea seal species, the grey seal, rarely suffer from parasitic bronchopneumonia (Chapter 2). Although Gosselin and Measures (1997) found *P. gymnurinus* in Canadian grey seals, live-stranded grey seals of the Dutch coast appear to have parasitic pneumonia caused exclusively by *O. circumlitus* (SRRC unpublished data).

Several factors could play a role, such as host-parasite compatibility, immune response to parasites, dietary intake, and food shortages affecting body condition.

Common seals may be a more suitable host for seal lungworms than grey seals. While parasites infect a wide variety of hosts, they often reach maturity in only a sub-set of them. Interspecific differences in infection levels may be related to morphological and/or physiological compatibility, affecting parasite growth and fecundity (Lagrue *et al.* 2011).

Good immune system functioning is key to controlling parasite infections (Wakelin 1996). Infection with these larvae could result in parasitic pneumonia when the immunological system is compromised. Pollution may play a role in the susceptibility of seals to parasitic infection. Current pollution levels were reported to remain high enough to exceed the levels at which immunosuppressive effects have been measured for seals (Ross 1995; Rijks 2008). Experimental studies that relate pollution to parasitic infections are scarce due to the complicated study design required (Sures 2008). Gendron *et al.* (2003) conducted an experimental study with leopard frogs which showed that exposure to a pesticide mixture increased the infection rate with lungworms. Furthermore, for glaucous gulls, a correlation was found between pollution levels and intensity of parasite infections (Sagerup *et al.* 2000). Similarly, Rohde (1984) reported on higher levels of ectoparasites in fish from polluted waters compared to fish from non-polluted waters. Little literature is available on the rate of disease caused by lungworms in common seals from different areas of the world. Gerber *et al.* (1993) reported for the California coast (1984-1990; n=227) that parasites were not a common finding in Pacific common seals admitted to rehabilitation. In a more recent study for the same area (Greig *et al.* 2014), rates of seals that stranded with parasites were not given, but only 32 out of 175 stranded common seals were of the age group (4 weeks - 1 year) in which lungworms are known to cause disease. It therefore appears that among Pacific common seals parasitic pneumonia is a much less frequent cause of disease than in European common seals. Further research would be of interest comparing parasite infestation levels in host populations in ecosystems with different levels of environmental pollution.

Next to contaminant-related immunosuppression, also the early age at which Wadden Sea common seals become infected (Chapter 8) may be important, as their immune systems may not yet be fully developed (Ross *et al.* 1993; Ross *et al.* 1994). Possibly, the immune system of common seals is less capable of fighting these lungworms than the immune systems of grey seals. It could be that common seals are more immunocompromised or that they are confronted with larvae at a younger age. Comparative studies of the immune systems of these two species are needed to understand why common seals are more susceptible to lungworm infections.

Also, dietary differences between common and grey seals could play a role. There may be differences in diet with respect to the occurrence of fish species that serve as parasite intermediate hosts. Interestingly, the hooknose was not found in the diet studies of grey seals undertaken in the UK (Hammond *et al.* 1994; Brown *et al.* 2012) and the Baltic (Lundström *et al.* 2007). If hooknose is indeed an important part of the *P. gymnuris*

lifecycle, this would fit with the absence of this lungworm species in grey seals. However, flatfish are part of the diet of both seal species. The search for the intermediate host of *O. circumlitus* should focus on species that serve as prey for both common and grey seals.

In addition to diet, the foraging area may be important for dietary intake. There is a difference in foraging area between common and grey seal pups. Common seals are born on sandbanks in the Wadden Sea and also in estuaries whereas grey seals pups are born on sandbanks on the border of the Wadden Sea and the North Sea. It is probable that fish in the Wadden Sea carry more larvae than fish from offshore waters due to the higher density of defecating seals. This may be especially true for the estuary of the Dollard, which is one of the main pupping areas of common seals in the Dutch Wadden Sea (Chapter 9).

Declining fish stocks and a lack of available fish may also play a role. Generally, common seals forage in coastal waters whilst grey seals forage further off-shore (Thompson *et al.* 1996). As a consequence, a much smaller range of foraging habitats is available for common seals. There are reports suggesting that fish stocks of the Wadden Sea may be declining (Tulp *et al.* 2008; Van der Veer *et al.* 2011; Van der Heij & Streefkerk 2014). Negative associations between host body condition and intensity of nematode infections were found in several wildlife studies, e.g. for baboons (Eley *et al.* 1989), geese (Shutler *et al.* 2012), and spoonbills (Sepulveda *et al.* 1994). It often remains unclear whether such associations are caused by the depressing effect of parasites on the host's body condition or alternatively whether an initial poor nutritional state made these hosts more susceptible to infection (Irvine *et al.*, 2006). Next to body condition, food shortages can also trigger a prey switching event. If the new prey item is heavily infected with parasites, this could result in an increased prevalence rate.

Conclusion

To conclude, we found *P. gymnurus* larvae in hooknose, common dab, and European plaice. This is the first record of *P. gymnurus* in hooknose. However, only L1s were found in this fish species, therefore it is not clear where this fish fits in the lifecycle of the worm. Future studies should focus on the hooknose, including experimental infections with *P. gymnurus*. In common with previous lifecycle studies on *Otostrongylus circumlitus*, larvae of this species were not found in any of the fish collected in this study. Future searches for the intermediate host of this nematode species should focus on prey species that serve as fodder for both juvenile common and grey seals.

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