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Heartworm (*Acanthocheilonema spirocauda*) and seal louse (*Echinophthirius horridus*) infections in harbour seals (*Phoca vitulina*) from the North and Baltic Seas

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ABSTRACT

The seal louse (*Echinophthirius* [*E.*] *horridus*) and the heartworm (*Acanthocheilonema* [*A.*] *spirocauda*) are parasites of harbour seal (*Phoca vitulina*). Little is known about the role of the seal louse as a potential vector and its role for the development and transmission of heartworm larvae to their final host, the harbour seal. The life-cycle of the heartworm is still not fully understood. For the presented study, findings of 1191 stranded harbour seals collected along the North- and Baltic Sea coast between 1996 and 2013 were examined. 4.4% (n = 53) of these harbour seals were infected with adult heartworms and 3.4% (n = 40) harbour seals carried seal lice. The highest prevalence and level of infection with adult heartworms (*A. spirocauda*) (9.3%) and seal lice (*E. horridus*) (8.9%) were found on yearling harbour seals (7–18 months) compared to neonate and adult seals. Investigating seal lice (n = 35) for larval heartworm stages one larvae was encountered in an ethanol-fixated seal louse. During a health monitoring survey of live harbour seals, 109 animals were captured and examined during spring and autumn between 2008 and 2014. Blood samples were taken and microfilariae were discovered in blood smears in 41% (n = 45) of the examined harbour seals. Yearling seals (n = 21) showed higher prevalence (86%) and level of infection with microfilariae than adults. Microfilariae were identified as *A. spirocauda* by sequencing the species-specific COI gene in 24 blood samples. The high prevalence of microfilariae of *A. spirocauda* in blood samples (41%) is in contrast to the low prevalence of mature infections/adult specimens in stranded seals (4.4%) investigated. Although rare parasites of seals, the recent increase in prevalence of heartworm and seal lice in stranded seals and the relatively high occurrence of microfilaria in the free-ranging population underscore the importance of further studies investigating the immunology of infections and their transmission pathways, as well as the epidemiology of both species.

1. Introduction

The seal louse (*Echinophthirius* [*E.*] *horridus*) and the heartworm (*Acanthocheilonema* [*A.*] *spirocauda*) are parasites of the harbour seal (*Phoca vitulina*). It is assumed that the hematophagous seal louse is essential for the development of microfilaria and transmission of the heartworm larvae to their final host, the harbour seal (Geraci et al., 1981). During their adaptation to the marine environment some parasite species of the harbour seal's terrestrial ancestors were lost, but presumably the heartworm *A. spirocauda* and seal louse *E. horridus* (Anoplura; Insecta) underwent a coevolution with their host and remained throughout its transmission to the aquatic milieu (Leidenberger et al., 2007). The life-cycle of the heartworm is still not fully understood and little is known about the role of the seal louse as a vector (Conlogue et al., 1980; Dunn and Wolke, 1976; Mehlhorn et al., 2002; Taylor et al., 1961). Harbour and grey seals (*Halichoerus grypus*) are two reproducing pinniped species in the North and Baltic Seas (Burns, 2002; Hammil, 2002; Siebert et al., 2012), and both populations have been increasing over the last years (CWSS, 2014; HELCOM, 2013). Two Phocine Distemper Virus (PDV) epidemics in 1988/89 and 2002 (Härkönen et al., 2006; Müller et al., 2004) reduced the population 40–60%, respectively. In 2014 an unusual mortality of European harbour seals occurred, resulting from infections with a novel influenza A H10N7 strain (Bodewes et al., 2015; Zohari et al., 2014).

Although they share the same habitat and prey species, grey seals apparently do not get infected by heartworms (Leidenberger et al., 2007; Measures et al., 1997) while both seal species are infected by seal lice (Durden and Musser, 1994). In contrast to cetaceans seals have retained an amphibian life-style (Scherf, 1963a,1963b) and rely on terrestrial habitat for reproduction and moulting (Boyd et al., 1999; Riedmann, 1990; Siebert et al., 2012). While parasitism is ubiquitous in wildlife and harbour seals are regularly infected by endo- and

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ectoparasites (Lehnert et al., 2007; Raga et al., 2002), most parasite species infecting harbour seals belong to endoparasitic nematodes of the respiratory and gastro-intestinal tract. The heartworm *A. spirocauda* is their only filarial parasite (Lehnert et al., 2007). The seal louse *E. horridus* is assumed to be a vector/intermediate host, because it has been shown to carry filaroid larval stages (Geraci et al., 1981). However, the evidence is restricted to a single study where the first three larval stages of the heartworm were shown to develop in the louse (Geraci et al., 1981). Subsequent studies failed to confirm the presence of heartworm larvae in seal lice. Therefore, mosquitos or simuliids as potential vectors (Dunn and Wolke, 1976) or transplacental transmission of microfilariae in seals has been discussed (see Leidenberger et al., 2007 for a review). The parasite host system could be assumed to be highly host-specific, but heartworms are found in several pinniped species, like harp, hooded and ringed seals from the arctic (Measures et al., 1997), as well as seal lice (Durden and Musser, 1994). The amphibious seal lice are one of the few insects in the ocean and developed claws to hold on during time in the water, use host sebum for insulation, and retain air within their spines during dives of their host (Leonardi et al., 2012; Mehlhorn et al., 2002; Messener et al., 1998). Seal lice are assumed to disperse by physical contact between host individuals during haul-out either vertically or horizontally (Leonardi et al., 2013). In terrestrial mammals, birds, and humans, insects often serve as vectors for infectious disease (Aspök, 2005), such as mosquitos in Malaria and sleeping sickness (Geiger et al., 2007) or ticks in Lyme borreliosis (Olsen et al., 1993). Similar parasite systems are known in dogs and their heartworm *Dirofilaria immitis*, which is transmitted by mosquito bites (Lai et al., 2000; Sacks et al., 2003). Infections in dogs have been successfully diagnosed using molecular tools to identify microfilaria from blood samples (Furtado et al., 2009; Mar et al., 2002). Lice have been described to transmit various organisms and diseases (Anderson, 2000; Hase, 1931) e.g. Typhus bacteria (*Rickettsia prowazekii*) in man (Hase, 1931) and *Acanthocheilonema reconditum* (*Linognathus setosus*) in dogs (*Canis lupus familiaris*) (Pennington and Phelps, 1969). Lice belonging to the suborder Amblycera and Ischnocera transmit two species of *Eulimdana* to birds (*Limosa fedoa*, *Numenius phaeopus*) (Bartlett, 1992). A novel alphavirus was isolated from the elephant seal louse (*Lepidophthirus macrorhini*) parasitizing southern elephant seals (*Mirounga leonina*) from Australia (La Linn et al., 2001). *Bartonella* spp. were found in harbour seals and their seal lice in the Netherlands (Morick et al., 2009). These examples show that marine mammal insect parasites can serve as vectors for infectious pathogens. Nevertheless, lice are rarely vectors of filarial worms (Geraci et al., 1981).

The aim of this study was to analyse prevalence and age-dependent infections of two related parasite species in harbour seals over time and to shed light on the life-cycle of heartworms and their potential vector, the seal louse. This is the first study that uses blood samples of wild-caught seals and molecular tools to assess the prevalence of *A. spirocauda* infection in a seal population and compares these results with necropsy findings of stranded seals with heartworm and *E. horridus* infections from more than a decade. The high prevalence of microfilariae in blood samples of live seals indicates the importance of new data about epidemiology and immunology of these relatively rare parasites in harbour seals, and underscores the relevance of molecular tools to study life-cycle characteristics in marine mammal parasites.

2. Material and methods

2.1. Health monitoring of stranded seals

Heartworms and seal lice were collected during necropsies performed in the frame of a health-monitoring on stranded harbour seals from the Baltic and North Seas off Germany during 1996–2013. All data investigated for this study originated from seals (n = 1191) that were examined as part of monitoring programmes to evaluate their health status (Siebert et al., 2007). Some animals found terminally ill were killed by gunshot by authorised seal rangers or by lethal injection according to animal protection laws by a veterinarian (Lehnert et al., 2007). Animals were dissected directly or after storage at − 20 °C for up to 6 months. Necropsies were performed according to Siebert et al. (2007). All organs were examined macroscopically and histologically. Seals were divided into age classes (AC): 'age class 0' [born and deceased in the same year (>6 months)]; 'age class 1' [seal born the previous year (up to 18 months)]; and 'age class 2' (animals >18 months). Three animals had no age classification and in four individuals the gender was not recorded. Parasites were collected during necropsies, preserved in 70% ethanol or frozen at −20 °C or −70 °C and identified microscopically after preparation in lactophenol. Macroscopic parasite infections were determined during necropsy semiquantitatively: none = no parasites, mild = mild infection, moderate = moderate infection, and severe = severe infection. Heartworms and seal lice were identified based on morphological characteristics of adults (Scherf, 1963a, 1963b; Anderson, 1992). Positive control DNA was isolated from adult specimens of *A. spirocauda* infecting harbour seals using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). Voucher specimens have been deposited in the Senckenberg Institute, Forschungsinstitut und Naturmuseum Frankfurt, Frankfurt, Germany (accession nos. SMF 17022; SMF-Pht 0001).

2.2. Lice dissection

From three individual seals (Supplementary Table 2), 35 adult seal lice that had been preserved in 70% ethanol were dissected using a binocular (Olympus SZ 61) at 40× magnification. 12 of these were kept in NaCl (saline solution) overnight prior to dissection. The abdomen was opened, the haemolymph was dried on a slide and the lice were dissected while being moistened with Ethanol or NaCl. All preparations were dried, fixed with 99% ethanol and stained with Giemsa and May-Grünwald (Pappenheim). After drying the slides were screened microscopically for larval nematode stages using 100× magnification (Olympus CX 41), measured (CellSens Entry software) and photographed (Olympus SC30 camera).

2.3. Health monitoring wild-caught seals

Free-ranging harbour seals (n = 109) were captured in the frame of a health-monitoring on two sandbanks (Lorenzensplate (LP) and Kolumbusloch (Kol) in the German Wadden Sea and on the Danish islands on Rømø (R) and Anholt (A) during spring (March/April) and autumn (August/September) from 2008 to 2014. Sex was determined and age judged by their weight and length. Medical examinations including haematology, blood chemistry and microbiology were performed, the weight, length and blubber thickness of the animals were taken. The seals were captured in nets and restrained during the examinations and before their release back into the sea as described by Hasselmeier et al. (2008).

Blood was taken from the epidural intravertebral vein (Dierauf and Gulland, 2001) and collected in ethylenediaminetetraacetic acid (EDTA) tubes. Blood smears were produced from frozen full blood or cruor that had been stored at −20 or −80 °C and the slides were stained with Giemsa and May-Grünwald (Pappenheim). After drying the slides were screened for microfilariae microscopically using 100× magnification (Olympus CX 41). The level of infection was determined semiquantitatively as none = no filarial stages, mild = mild infection, moderate = moderate infection, severe = severe infection (see Supplementary material Table 1). From nine fresh blood samples (2011/2013) filarial stages were measured for length (n = 46) and width (n = 20) (CellSens Entry software) and photographed (Olympus SC30 camera).

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2.4. Molecular techniques

Genomic DNA from EDTA full blood samples or EDTA cruor was isolated using a QIAamp Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Mitochondrial DNA of COI (Cytochrome c oxidase subunit I) was amplified by PCR using primers designed from the COI sequence for *A. spirocauda* published in GenBank (accession no.: HF583266.1). Oligonucleotide primers were: 5′- GGTCCTGGGAGT AGCTGAAC -3′ and 5′- ATGATGGCCCCACACAGAAG -3′. The PCR started with an initial step at 94 °C for 3 min, followed by 39 cycles of: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min. It ended with a 5 min step at 72 °C. Primer concentrations were 20 pmol/μl and Taq polymerase (Applied Biosystems, Darmstadt, Germany) was used. Sequencing reactions were performed at Seqlab Sequencing Laboratories (Göttingen, Germany) for each PCR product twice (forward & reverse). Nucleotide sequences were edited and aligned using Chromas (Chromas lite 2.1.1.) and DNAstar (version 5.07/5.52) software. As a positive control DNA from adult *A. spirocauda* and negative controls without template were included in PCR reactions.

2.5. Statistical analyses

To identify the factors that affect age and gender related patterns in parasite infection a logistic regression was used (Venables and Ripley, 1999) where the dependent variable was a binary variable (0 or 1). It was 1 if an animal was infected with a parasite, 0 otherwise. As explanatory variables seal louse infection (binary (0,1)), sex, age class and their interaction were used. The risk of seal louse infection by sex, age class and their interaction was modelled. Model selection was accomplished by AIC-values (Venables and Ripley, 1999). All analyses were conducted in R version 3.1.3 (R Core Team, 2015).

3. Results

3.1. Health monitoring of stranded seals

Of 1191 examined seals collected between 1996 and 2013, most were young-of-the-year seals (n = 831; AC0), 225 individuals were yearlings (AC1) and 132 seals were adults (AC2). 53 seals were infected with mature *A. spirocauda* in the heart, 40 seals had seal lice (*E. horridus*) infections and 11 of those seals had a mixed/co-infection. All infected seals came from the North Sea coast, and the sex ratio was almost equal (females = 596; males = 591). Heartworm and seal louse prevalence varied over the years (Fig. 1).

In 11 of 82 infected seals (13%) and in five of the 17 (30%) study years heartworm and seal louse occurred together in a co/mixed infection. In the years 1996, 1998, 2004 and 2006 only seal lice were found, in 1999, 2010 and 2011 only heartworms were detected. In 1997, 2000, 2001, and 2007 both species were identified in different individuals and in five study years (2002, 2008, 2009, 2012 and 2013) they occurred in mixed infections (Fig. 1).

Prevalence between 1996 and 2012 was 2.6% (heartworm) and 2.6% (seal louse; n = 1100). The number of investigated animals per year during that time was 21–198. In 2013 heartworm prevalence was 26.3% and seal louse prevalence 15.4% (n = 91).

3.1.1. Heartworm infections

53 seals (4.4%) were infected with adult *A. spirocauda*. Of these animals, 32 were male (60%) and 21 female seals (40%). Age class 0 seals (n = 831) had a 3.5% (n = 29) prevalence of heartworm infection, AC1 (n = 225) had a 9.3% (n = 21) prevalence of infection and AC2 (n = 132) had a 2.3% (n = 3) prevalence of infection with heartworm (Fig. 2).

Of the 29 infected seals in AC0 86% (n = 25) were infected mildly, 7% (n = 2) moderately and 7% (n = 2) were infected severely with heartworms. In AC1 57% (n = 12) of the infected seals had a mild infection, in 33% (n = 7) a moderate and in 10% (n = 2) a severe infection with adult heartworms was found. In AC2 67% (n = 2) of the infected seals had a mild, and 33% (n = 1) a severe infection. Heartworm infections observed in these animals were not associated with heart lesions (Lehnert et al., 2007), although one heartworm-infected animal had a perforation of the right ventricle. Frequently, co-infections with lungworms were present.

No significant difference in heartworm prevalence between females and males was found (Table 4). However, louse infection greatly influenced the risk of heartworm infections (Tables 4 and 5). If a seal showed a seal lice infection the risk of a heartworm infection was found to be 7.5 times higher than without lice present (Table 5). Prevalence of infection with heartworm and seal lice between age classes differed significantly between AC0 and AC1 (p = 0.01), as well as between AC1 and AC2 (p = 0.04), but not significantly between AC0 and AC2. When accounting for both, age effects and seal lice infection, the effect of age class decreases (compared to the model with only age effect (Model 5), see Table 4), as

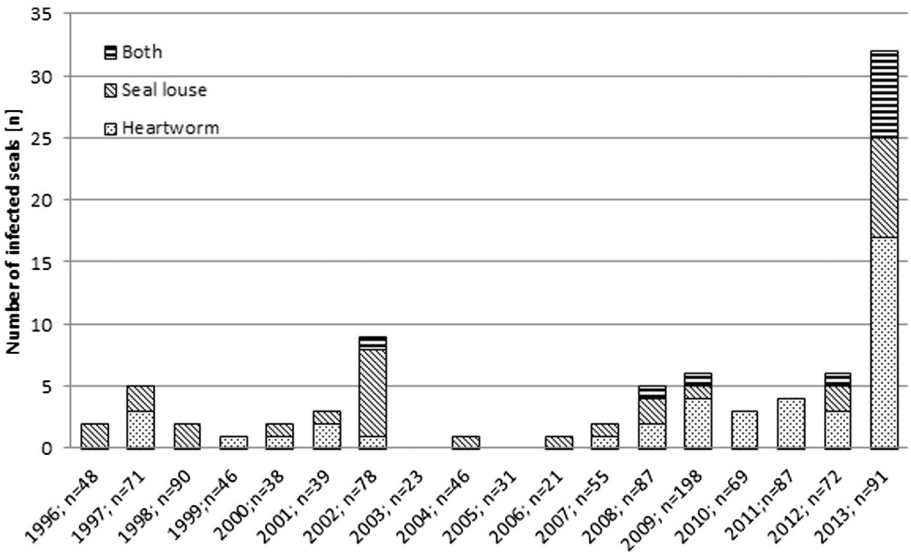

Fig. 1. Prevalence of mature heartworm *A. spirocauda*, seal louse *E. horridus* and co-infections in stranded harbour seals (*P. vitulina*) (n = 1191) between 1996–2013.

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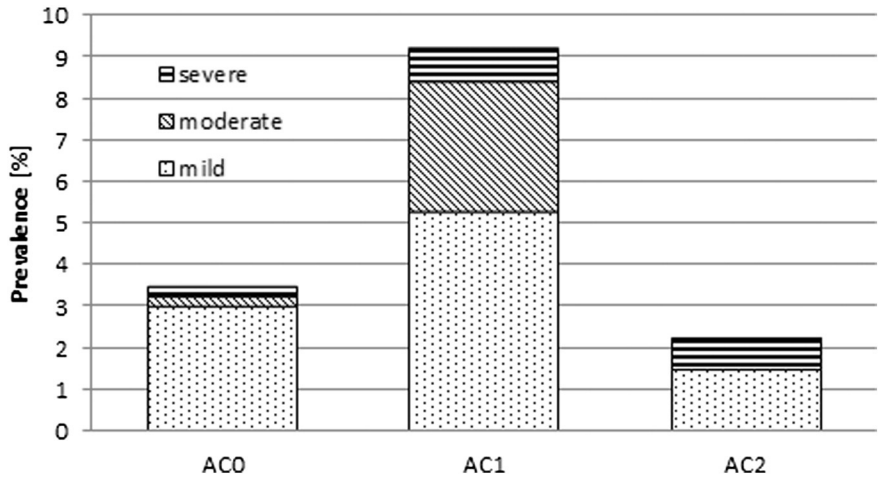

Fig. 2. Prevalence (%) and level of infection (mild, moderate, severe) of heartworm *A. spirocauda* infection in age classes of stranded harbour seals (*P. vitulina*) (n = 1191); number of investigated seals per age class: AC0 (age class 0) = 831, AC1 = 225, AC2 = 132 seals.

lice infection strongly explained heartworm infection and lice infection is higher in AC1. There is no significant interaction between age class and lice infection (Table 4).

3.1.2. Seal lice infections

Measurements of adult lice, nymph and nit stages are shown in Table 1.

40 seals (3.4%) were infected with *E. horridus*. 21 male (54%) and 19 female seals (46%) were infected. AC0 seals (n = 831) had a 1.9% (n = 16) prevalence of seal lice infection, AC1 (n = 225) had an 8.9% (n = 20) prevalence of infection and AC2 (n = 132) had a 3% (n = 4) prevalence of infection with seal lice (Fig. 3).

81% (n = 13) of the infected seals in AC0 were infected mildly with seal lice and 6.25% (n = 1) had a moderate and 12.5% (n = 2) a severe infection. 30% (n = 6) of the infected seals in AC1 had a mild, 25% (n = 5) a moderate and 45% (n = 9) a severe infection with seal lice. In AC2 25% (n = 1) of the infected animals had a mild and a moderate infection, 50% (n = 2) had a severe infection with seal lice. Four animals with severe seal lice infections had anaemia, in 6 animals with severe seal lice infections endo- and ectoparasitosis contributed to illness and mortality. AC1 had a significantly higher prevalence of lice infection than other age classes (p-value <0.001). It is about 2.7 times higher than for AC0 and 4 times higher than AC2. There is no significant difference between AC0 and AC2 (p-value = 0.412) and no differences in seal lice infections between female and male seals.

Table 1
Measurements of *Echinophthirius horridus* adult specimens, nymph and nit stages.

E. horridus	Number [n]	Length [mm]	Min.–max. [mm]	References
Male	27	2.58	2.15–2.94	This study
	50	2.36	2.11–2.60	Scherf (1963a,b)
	150	2.12	1.75–2.48	Beder (1990)
Female	36	3.05	2.25–3.66	This study
	50	2.61	2.34–3.03	Scherf (1963a,b)
	171	2.33	1.70–2.92	Beder (1990)
Nymph 1	28	1.21	1.06–1.34	This study
	50	2.12	0.97–1.25	Scherf (1963a,b)
	34	2.13	0.78–1.38	Beder (1990)
Nymph 2	19	1.46	1.25–1.62	This study
	50	1.49	1.29–1.62	Scherf (1963a,b)
	42	1.44	1.20–1.83	Beder (1990)
Nymph 3	33	1.86	1.66–2.07	This study
	50	1.91	1.74–2.15	Scherf (1963a,b)
	53	1.79	1.58–2.13	Beder (1990)
Nit length	36	0.967	0.849–1.123	This study
	–	0.923	–	Scherf (1963a)
Nit width	34	0.503	0.365–0.621	This study
	–	0.464	–	Scherf (1963a)

3.1.3. Lice dissections

One larva was encountered in an ethanol-fixated seal louse (Supplementary Fig. 1). It was 274.68 μm in length and 7.93 μm in width. The larva in the seal louse came from a female AC1 seal that had a severe seal louse and no heartworm infection (Supplementary Table 2, seal number: 33).

3.2. Health monitoring of wild-caught seals

3.2.1. Blood samples

Harbour seals (n = 109) were captured on two sandbanks (Lorenzensplate (LP; n = 68) and Kolumbusloch (Kol; n = 28)) in the German Wadden Sea (n = 96) and on the Danish islands on Rømø (R) (n = 8) and Anholt (A) (n = 5) during spring (March/April) and autumn (August/September) from 2008 to 2014. 88 animals were adults of AC2 (females n = 39; males n = 49) and 21 animals were yearlings of AC1 (females n = 12; males n = 9), judged by their weight and length. Of 109 investigated seals (females = 51; males = 58), 21 belonged to AC1 and 88 to AC2. No lice were found on the seals during the catches, but no systematic or dedicated search was possible. 45 seals (41%) had microfilariae in their blood smears, 18 (35%) were females and 27 (53%) were males. 18 infected seals belonged to AC1 (86%) and 27 (31%) were AC2. The prevalence of microfilariae in blood smears varied between 11% and 57% during 2008 and 2014 (Table 2). In 30 animals a mild infection with microfilariae was found, 11 had a moderate infection and four a severe infection. MF had a blunt head and spiked tail

Table 2
Prevalence of microfilariae (MF) in blood smears (n = 109) between 2008–2014.

Month/year	Seals investigated [n]	MF present in blood smears [n]	Prevalence [%]
Apr 08	21	12	57
Sep 09	10	5	50
Apr 10	9	1	11
Sep 10	13	4	31
Apr 11	14	8	57
Mar 12	10	5	50
Apr 13	18	8	44
Sep 13	9	1	11
Apr 14	5	1	20

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Table 3
Measurements of microfilariae (MF) (n = 46) in blood smears of wild-caught harbour seals (*Phoca vitulina*) compared with previous studies.

MF [n]	Length [mm] ± SD	Min.–max. [mm]	MF [n]	Width [mm] ± SD	Min.–max. [mm]	Reference
46	229.05 ± 10	203.37–250.38	20	5.16 ± 0.5	4.22–5.93	This study
–	235 ± 10	–	–	5.4	–	Taylor et al. (1961)
40	286 ± 10	266–302	40	5.3 ± 0.5	4.4–6.2	Geraci et al. (1981)

(Supplementary Fig. 2), their length was 229.05 μm (± 10 μm) and their width 5.16 μm (± 0.5 μm) (see Table 3).

Overall, prevalence of microfilariae in blood smears of free-living seals was 41%. Prevalence of adult *A. spirocauda* and seal lice infections in stranded seals submitted to postmortem investigations was 4.4% (mature *A. spirocauda*) and 3.4% (seal lice, respectively).

There was no significant difference in infections with microfilaria between male and female harbour seals. A significant difference in prevalence of microfilaria between AC1 and AC2 was found. AC1 had in general a higher prevalence (p-value <0.001) of MF infections. Females had a lower prevalence (p-value = 0.098) than males. The highest prevalence and level of infection of microfilariae, adult heartworm and seal louse was observed in AC1 (Fig. 4) when comparing results from blood smears of wild-caught seals to stranded animals subjected to post mortem investigations.

3.2.2. Molecular analyses

Genomic DNA was isolated from 45 blood samples of seals that had microfilariae in their blood smears (see Supplementary Table 1). PCR reactions were performed, using a species-specific COI primer. 27 PCR products produced a distinct band of the expected base pair length (~500 bp) in electrophoresis. Sequencing reactions of PCR products that yielded a band were performed and 24 PCR products of 24 individual seal blood samples yielded a good-quality sequence of 340–450 bp, that was identical to the *A. spirocauda* published sequence of the COI gene for cytochrome oxidase subunit 1 (accession no.: HF583266.1) in GenBank. All sequences were aligned and when blasting the 431 bp consensus sequence of our derived sequences a 99% identity was found with *A. spirocauda* COI gene (accession no.: HF583266.1).

4. Discussion

4.1. Health monitoring of stranded seals

Heartworm *A. spirocauda* and seal louse *E. horridus* seldom parasitise harbour seals in the North Sea and occur with low prevalence and levels of infection compared to other parasite species like anisakid stomach worms (e.g. *Pseudoterranova decipiens*) or nematodes from the respiratory tract (Metastrongyloidea), that show prevalences from 60–80% (Lehnert et al., 2007; Siebert et al., 2007) and are often found in severe infections. Consequently, heartworm and seal louse infection seldom cause severe disease or mortality in harbour seals from this area (Lehnert et al., 2007; Siebert et al., 2007). This is supported by findings in pinnipeds from the Atlantic coast of Canada (Measures et al., 1997) where heartworm prevalence was low. Apparently there were 65% infected harbour seals found in the Netherlands (van den Broek, 1963) and 32% (Claussen et al., 1991), 25% (Borgsteede et al., 1991) and 11.4% (Lunneryd, 1992), respectively, found in North and Baltic Sea harbour seals who died during the 1988/89 phocine distemper virus (PDV) epizootic. Heartworms had no apparent impact (nor associated lesions) on the health status of their hosts, although some lesions in the heart associated to *A. spirocauda* have been described (Dunn and Wolke, 1976; Measures et al., 1997). Seal lice can cause alopecia, skin lesions and anaemia, but only seldom induce severe lesions with resulting in a higher mortality (Conlogue et al., 1980; Lehnert et al., 2007). It can be assumed that animals suffering from severe infections of e.g. lice are most likely immune compromised or were in an otherwise poor health or nutritional status prior to accumulating this severe parasite burden (Thompson et al., 1998). The results from the post mortem investigations show that prevalence is varying strongly throughout the study period. This may be related to the variable number of animals examined per year and because lice may have been lost after death (Thompson et al., 1998), before the animals were collected for post mortem investigations. Mixed infections of heartworm and seal lice in one host have been described (Conlogue et al., 1980; Geraci et al., 1981; Taylor et al., 1961; Wülker, 1930) and a significant positive correlation between both was found (Daley & Fallace, 1989), supporting the findings from this study. Also density dependent factors and immune suppression influencing the transmission of lice between seals need to be taken into account, e.g. before the PDV epidemics. Prevalence and intensity of infection with seal lice in pinnipeds reportedly differ among regions (Leonardi and Palma, 2013), with 41% infections in harbour seals reported from the Wadden Sea (Wipper, 1974), 39% in Scottish waters (Thompson et al., 1998) and 45% on the Pacific coast of North America (Dailey and Fallace, 1989). Age-dependent infections with immature seals carrying highest burdens (Thompson et al., 1998; Wipper, 1974) are common. Several studies reporting heartworm in harbour seals did not report or find *E. horridus* (Borgsteede et al., 1991; Lunneryd, 1992). Heartworms mainly infect immature seals (Dunn and Wolke, 1976; Lunneryd, 1992), and after infection the prevalence seems to decrease with increasing age of the host. This finding is supported by previous studies (Borgsteede et al., 1991; Claussen et al., 1991; Dunn and Wolke, 1976; Lunneryd, 1992). Our results show an age dependence of infections with *A. spirocauda* and *E. horridus* that seem to be most prevalent and with highest levels in AC1, both in stranded and live-caught seals although no AC0 seals could be investigated for microfilaria in this study. This may reflect an influence of the immune status of the animals. Age-dependent infections were reported from the Wadden Sea after the PDV epidemic in 1988/89 (Claussen et al., 1991), Denmark (Lynneryd et al., 1992), the Netherlands (Borgsteede et al., 1991) and other geographical regions (Delyamure & Treshev, 1966; Measures et al., 1997). The encountered age dependence in prevalence and level of infection with both parasite species supports that they are

Table 4
Models for estimating the proportion of individuals infected by heartworm and the corresponding AIC-values. Null deviance was 433.5.

Model	Model components	Residual deviance	AIC	ΔAIC
1	Prevalence lice + age group	399.5	407.5	0
2	Prevalence lice * age group	396.3	408.3	0.8
3	Prevalence lice + age group + sex	396.6	408.6	1.2
4	Prevalence lice	407.6	411.6	4.1
5	Age group	419.80	425.8	18.4
6	Age group + sex	417.32	427.3	19.9
7	Age group * sex	412.88	428.9	21.4
8	Sex	430.64	436.6	29.2

Table 5
Best model explaining proportion of individuals infected by heartworm using GLMs and AIC for model selection.

Variable	Coefficient	Std. error	p-Value
Intercept	−2.63	0.26	<0.001
Prevalence lice	2.06	0.4	<0.001
AG0	−0.8	0.3	0.01
AG2	−1.3	0.64	0.04

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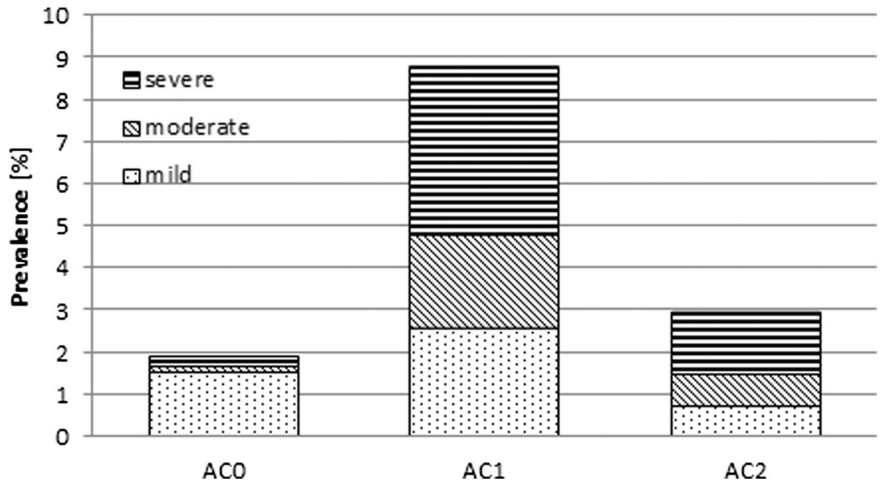

Fig. 3. Prevalence (%) and level of infection (mild, moderate, severe) of seal louse (*E. horridus*) infection in age classes of stranded harbour seals (*P. vitulina*) (n = 1191); number of investigated animals per age class: AC0 (age class 0) = 831, AC1 = 225, AC2 = 132 seals.

associated and may suggest that the transmission of seal lice and heartworms occurs generally horizontally between immature seals on land. Additionally, the life cycle of echinophthiriids is associated with host behaviour and limited by the time that seals spend hauling out. Immature seals spend more time ashore and therefore lice probably reproduce on them, resulting in higher infections. In South American sea lions (*Otaria flavescens*), from Patagonia, the transmission of *Antarctophthirus microchir* (Echinophthiriidae) has been shown to occur vertically from mothers to pups (Leonardi et al., 2013). There is data on morphology of adult *E. horridus* in the literature (see Table 1) and the results shown in this study support previous findings (Beder, 1990; Scherf, 1963a,1963b). No differences in infections between the sexes were obvious in seal lice or heartworm, as described in previous studies (Dailey & Falace, 1992; Thompson et al., 1998).

4.1.1. Lice dissections

The detection of a larval stage in a seal louse may support the hypothesis that heartworms potentially use seal lice as intermediate hosts for development and as a possible vector for their transmission to harbour seals. The larval stage found was longer and thicker than the microfilariae found in harbour seal blood smears in this study. The L1 stage is described as short and thick ‚sausage' without inner structures that develops head and tail shortly before moulting into L2 (Geraci et al., 1981). L2 are described as considerably longer (923 ± 202 μm) and thicker (29.7 ± 3.2 μm) (Geraci et al., 1981) with inner structures like oesophagus and intestine visible as in the larval stage found in the seal louse in this study (Supplementary Fig. 1). Based on the similarity in size and the description of L1 and L2 in previous studies it is assumed that the stage encountered here may be a late L1 stage. Obligate intermediate hosts like the seal louse often accommodate only small amounts of parasitic developmental stages and therefore cause low infection rates in the host (Conlogue et al., 1980). Because all developmental stages suck blood (Geraci et al., 1981; Lauckner, 1985), also nymph stages could possibly serve as intermediate hosts. This indicates that sucking lice (Echinophthiriidae) may act as vectors for filarial nematodes, although lice are rarely used for transmission compared to other insects (Geraci et al., 1981). Certainly, dissections of a higher number of fresh seal lice and nymphs are required and a screening of lice by an in situ hybridization using species-specific probes for *A. spirocauda* may increase the detection rate of larval stages in lice.

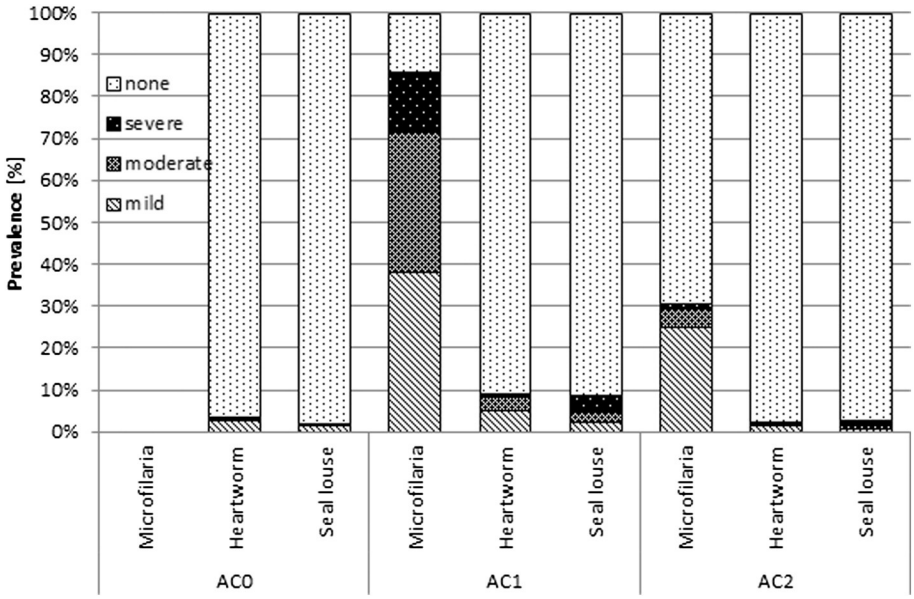

Fig. 4. Prevalence and level of infection with microfilaria, adult heartworm (*A. spirocauda*) and seal louse (*E. horridus*) in age classes 0, 1 and 2 (microfilariae in AC0 = not investigated).

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4.2. Health monitoring of wild-caught seals

Larval stages in blood smears showed specific morphology typical for microfilariae (Supplementary Fig. 2) with a blunt head, pointed tail (Dunn and Wolke, 1976), exceedingly slender (Taylor et al., 1961) and without inner structure. There is little actual data on morphology of microfilariae in the literature (see Table 3). The measurements performed in this study seem to be in concordance with those of previous reports (Geraci et al., 1981; Taylor et al., 1961), and variance may be due to differences in fixation techniques and measuring methods that were not described in detail in the two previous studies. The size and shape of the microfilarial stages support that they are not to be confounded with nematode L1 or third stage larvae of e.g. metastrongyloid lungworms or anisakid stomach nematodes (Dailey, 1970; Houde et al., 2003; Measures, 2001). Microfilariae in blood samples were reported also in four Atlantic harbour seals (Dunn and Spotte, 1974) originating from Sable Island, Nova Scotia, were MF were identified "tentatively" as *A. spirocauda*. Another report described occlusion of the pulmonary artery and acute vasculitis due to microfilariae in the vascular system, and morphological lesions in the hepatic and pulmonary system due to migrating filariae after the same animals had died in captivity in Connecticut, USA (Dunn and Wolke, 1976). The identification of MF to the species level by amplification and sequencing of species-specific markers proved to be an unequivocal way to identify the species of larval nematode encountered in this study. Some PCR reactions in this study using the species-specific primers yielded no PCR product or electrophoretic band ($n = 18$) and some yielded no sequence ($n = 3$). In these cases the minute amount of DNA isolated was probably not sufficient for PCR reactions or no filarial stages were included in the blood extracted from the sample for DNA isolation. Molecular tools have been shown to be valuable in identifying larval parasites, in which morphological characteristics are not sufficient for species determination (Campbell et al., 1995; Chilton et al., 1995; D'Amelio et al., 2000). The number of animals caught for the health-monitoring of live harbour seals was predominated by adult individuals (AC2). Blood smears of immature seals (AC1) showed the highest prevalence and level of infection with microfilariae. The encountered age dependence in prevalence and level of infection may show that the transmission of seal lice and heartworms occurs generally horizontally between immature seals during haul-out. Although prevalence of both *A. spirocauda* and *E. horridus* in stranded seals increase strongly in 2013, these results clearly contrast the prevalence of microfilariae (41%) found in blood smears of wild-caught seals subjected to medicals. This may be due to the age bias in wild-caught versus stranded seals — in both groups some age classes are underrepresented and not evenly distributed and study periods differ (seven years in live seals vs. 17 years in stranded seals). The higher prevalence of microfilariae in live-caught seals points towards more heartworm infections in seals then previously assumed from interpreting data from stranded animals. In the future, blood smears of neonate seals (AC0) should be tested for microfilarial stages and potential other intermediate hosts (mosquitos) screened for larval filaroids.

5. Conclusion

A. spirocauda and *E. horridus* are rare parasites compared to other species infecting harbour seals in the North and Baltic Sea and seldom seem to have a detrimental effect on host fitness. Both parasite infections are age dependent and predominantly occur in AC1. Heartworm prevalence has clearly decreased from the last studies in this area during the 1988/89 PDV epidemic (Borgsteede et al., 1991; Claussen et al., 1991; Lunneryd, 1992). According to Essink et al. (2005) seal louse prevalence has decreased from 15% in 1980 to 1.5% in 2000 in the Netherlands. Nevertheless, the recent increase in prevalence of heartworm and seal lice in stranded seals and the relatively high occurrence of microfilaria in the free-ranging population underscore the importance of further studies investigating the immunology of infections and their transmission pathways. The epidemiology of both species in relation to anthropogenic impacts on the marine environment and their hosts, like influences of environmental toxicants and immune suppression should be examined. The harbour seal population in the North Sea is increasing towards carrying capacity and experts were expecting a new virus epidemic soon. In the autumn of 2014 a novel influenza virus (H10N7) caused a 10–15% mortality (Bodewes et al., 2015) in the population. Consequently, the dynamics between population density and parasite dispersal need to be taken into account, especially when observing higher heartworm prevalence in seals during the 1988/89 PDV epizootic (Borgsteede et al., 1991; Claussen et al., 1991; Lunneryd, 1992), when harbour seal population numbers were high, and the increase of heartworm and seal lice prevalence in seals from this study in 2013. The rare host–parasite-system analysed in this work is an exception and can be seen as a relic of the seal's terrestrial ancestor's parasite fauna considering their amphibian *modus vivendi*. Molecular techniques can facilitate the identification of transmission pathways in host–parasite-interactions and improve the understanding of their life-history and ecology.

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