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**Prevalence, impact and molecular characterisation of
parasites in harbour porpoises and harbour seals**

Dissertation

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SUMMARY

Parasitism is ubiquitous in wildlife, but little is known about the dynamics of parasitic infections and associated diseases in marine mammals. This thesis aimed to characterise the parasite fauna of harbour porpoises (*Phocoena phocoena*) and harbour seals (*Phoca vitulina*) to assess the impact on their health status and to improve the knowledge about the life history of their lungworm species.

Parasitological investigations were carried out on harbour porpoises originating from the German North and Baltic Seas and the Norwegian North Sea. The parasites found in porpoises originated mainly from the respiratory tract and comprised four pseudaliid nematodes (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*), one ascaridoid nematode (*Anisakis simplex*) and two trematodes (*Campula oblonga*, *Pholeter gastrophilus*). The finding of *H. invaginatus* was the first geographic record from German waters. Differences were found in the severity of the parasitic infection between stranded and bycaught animals as well as between porpoises from different areas. A correlation was shown between parasitic infection and observed lesions. Lungworm prevalence and intensity of infections increased with age suggesting horizontal transmission through the food web. The data also indicated that infections were accumulated with age and long-lived.

The occurrence of whale-lice, *Isocyamus delphinii* (Crustacea: Amphipoda: Cyamidae), infestation was described in a stranded harbour porpoise. The emaciated animal had multiple, partially scarred wounds to which numerous small crustaceans were attached. The whale-lice showed a significant sexual dimorphism and their population was male biased. The lesions in the lung were found to be the likely cause of death in this porpoise, and the ectoparasites were not diagnosed to be a major health threat to the animal. The skin wounds' origin remained unclear, but the lice seemed to be closely associated and dependent on the wounds of this host. In this first and unusual occurrence of *I. delphinii* on a harbour porpoise from German waters, the transmission was probably interspecific and skin wounds were likely the necessary means for the parasites to attach and remain on a porpoise.

Eight species of parasites were collected from harbour seals from the German North Sea. Two anisakid nematodes (*Pseudoterranova decipiens* (sensu lato), *Contracaecum osculatum*

(sensu lato) from the stomach, *Otostrongylus circumlitus* (Crenosomatidae) and *Parafilaroides gymnurus* (Filaroididae) from the respiratory tract, one filaroidid nematode (*Acanthocheilonema spirocauda*) from the heart, two acanthocephalans *Corynosoma strumosum* and *C. semerme* (Polymorphidae) from the intestine and the ectoparasite *Echinophthirius horridus* (Anoplura, Insecta) were found. Lungworm infection was the most prominent parasitological finding and secondary bacterial bronchopneumonia the most pathogenic lesion correlated with the parasites. Heavy nematode burdens in the respiratory tract were highly age-related and more frequent in young seals. A positive correlation was observed between high levels of pulmonary infection and severity of bronchopneumonia. The prevalence of lungworms in this study was higher than in seals that died during the 1988/89 Phocine Distemper Virus epidemic, and prevalence of acanthocephalans and heartworms had decreased compared to findings from the die-off. The metastrongyloid nematodes of porpoises and seals from German waters parasitising the respiratory tract were especially pathogenic and often caused secondary bacterial infections. In spite of the importance of lungworm disease for the health of wild populations and their clinical and epidemiological significance, the life cycle and biology of lungworms in the marine environment is still largely unknown. The ITS-2 (rDNA) region of lungworms parasitising porpoises and seals were sequenced to characterise the different species. Molecular markers to identify larval nematode stages via *in-situ* hybridisation in tissues of porpoise and seal prey were developed. Potential intermediate hosts from the North Sea were screened for larval stages of lungworms using the species-specific traits of the ITS-2. Histology and *in-situ* hybridisation of tissue samples of flatfish showed larvae within the intestinal wall. Using larval ITS-2 nucleotide sequences the nematode stages were identified as *P. inflexus* and *P. gymnurus*. Experimental infections of turbot (*Psetta maxima*) with live seal lungworm larvae (*O. circumlitus*) revealed second and third-stage larvae of *O. circumlitus* in their intestinal tract. The results elucidated the life cycle of metastrongyloids of porpoises and seals and showed that they use vertebrate intermediate hosts.

This thesis discovered new aspects of the life history of lungworm parasites of porpoises and seals by combining novel molecular techniques with methods from traditional parasitology. New information on parasite species and associated diseases in porpoises and seals improved the knowledge about the biology of parasites and hosts and our understanding of infection patterns.

ZUSAMMENFASSUNG

Parasitismus bei Wildtieren ist allgegenwärtig, zur Dynamik von Parasiteninfektionen und damit assoziierten Erkrankungen in marinen Säugetieren ist jedoch wenig bekannt. In dieser Arbeit wurde die Parasitenfauna von Schweinswal (*Phocoena phocoena*) und Seehund (*Phoca vitulina*) charakterisiert und ihr Einfluss auf den Gesundheitszustand der Wirte abgeschätzt. Zusätzlich sollte der bisher unbekannte Lebenszyklus ihrer Lungenwurmspezies erforscht werden. Parasitologische Untersuchungen wurden an Schweinswalen aus der deutschen Nord- und Ostsee und norwegischen Gewässern durchgeführt. Parasiten kamen hauptsächlich im Respirationstrakt vor und bestanden aus vier pseudaliiden Nematodenarten (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*), einem ascaridoiden Nematoden (*Anisakis simplex*) und zwei Trematoden (*Campula oblonga*, *Pholeter gastrophilus*). *H. invaginatus* wurde zum ersten Mal in deutschen Gewässern beschrieben. Unterschiede in der Schwere des Befalls zwischen gestrandeten und beigefangenen Tieren sowie zwischen Schweinswalen aus verschiedenen Gebieten traten auf. Eine Korrelation zwischen Parasitenbefall und Gewebeläsionen in den betroffenen Organen konnte gezeigt werden. Lungenwurmprävalenz und die Intensität des Befalls nahm mit dem Alter der Wirtstiere zu, was auf eine horizontale Übertragung der Parasiten über das Nahrungsnetz schließen lässt. Die Daten ließen darauf schließen, dass die Infektionen langlebig waren und sich mit dem Alter akkumulierten.

Das Vorkommen von Walläusen, *Isocyamus delphinii* (Crustacea: Amphipoda: Cyamidae), auf einem gestrandeten Schweinswal wurde beschrieben. Das abgemagerte Tier hatte mehrere, zum Teil vernarbte Wunden, an denen zahlreiche kleine Crustaceen saßen. Die Walläuse zeigten einen signifikanten sexuellen Dimorphismus und ihre Population wurde von männlichen Tieren dominiert. Die Läsionen in der Lunge des Tieres wurden als Todesursache und die Ektoparasiten nicht als Gefahr für den Gesundheitszustand des Wirtes angenommen. Die Ursache der Hautwunden blieb unklar, aber die Walläuse schienen mit den Wunden assoziiert und von ihnen abhängig zu sein. Bei diesem ersten ungewöhnlichen Vorkommen von *I. delphinii* auf einem Schweinswal aus deutschen Gewässern fand die Übertragung möglicherweise interspezifisch statt und die Hautwunden waren wahrscheinlich die Voraussetzung für die Walläuse um auf diesem Wirt zu überleben.

Acht Parasitenspezies wurden in Seehunden aus der deutschen Nordsee isoliert: Zwei anisakide Nematoden (*Pseudoterranova decipiens* (sensu lato), *Contraecaecum osculatum* (sensu lato) aus dem Magen, *Otostrongylus circumlitus* (Crenosomatidae) und *Parafilaroides gymnurus* (Filaroididae) aus dem Respirationstrakt, ein filaroidider Nematode (*Acanthocheilonema spirocauda*) aus dem Herz, zwei Acanthocephalen *Corynosoma strumosum* und *C. semerme* (Polymorphidae) aus dem Darm und der Ektoparasit *Echinophthirius horridus* (Anoplura, Insecta). Lungenwurminfektionen stellten den häufigsten parasitologischen Befund dar und sekundäre bakterielle Bronchopneumonie die pathogenste Läsion, die mit den Parasiten korreliert war. Schwerer Parasitenbefall war altersabhängig und besonders häufig in jungen Seehunden. Die Lungenwurmprävalenz in dieser Studie war höher als bei Seehunden, die während der Staupeepidemie 1988/89 starben, hingegen hatte die Prävalenz von Acanthocephalen und Herzwürmern abgenommen.

Die metastrongyliden Nematoden von Schweinswal und Seehund waren besonders pathogen und verursachten häufig sekundäre bakterielle Infektionen. Trotz ihrer Bedeutung für den Gesundheitszustand wilder Populationen und ihrer klinischen und epidemiologischen Bedeutung ist ihr Lebenszyklus im Meer weitgehend unbekannt. Die ITS-2 (rDNA) Region der Lungenwürmer von Schweinswalen und Seehunden wurde sequenziert, um die verschiedenen Spezies zu charakterisieren. Die spezies-spezifischen Eigenschaften der Sequenzen wurden genutzt um potentielle Zwischenwirte auf Larvenstadien hin zu überprüfen. Molekulare Marker zur Identifizierung von Larvenstadien wurden entwickelt. Histologie und *in-situ* Hybridisierung zeigten Nematodenlarven in der Darmwand von Plattfischen, die Larven wurden anhand ihrer ITS-2 Region als *P. inflexus* und *P. gymnurus* identifiziert. Experimentelle Infektionen von Steinbutt (*P. maxima*) mit lebenden *O. circumlitus* Larven ergaben Zweit- und Drittlarven im Darmtrakt der Fische. Die Ergebnisse beleuchten den Lebenszyklus der Lungenwürmer von Schweinswalen und Seehunden und zeigen, dass sie vertebrierte Zwischenwirte für ihre Übertragung nutzen. Diese Arbeit deckte neue Aspekte der Biologie von Parasiten mariner Säuger auf, indem sie neue molekulare Techniken mit traditioneller Parasitologie verknüpfte. Informationen über Parasitenspezies und assoziierte Krankheiten in den Wirtstieren verbesserte unser Wissen über Parasit-Wirt Beziehungen und unser Verständnis der Infektionsmuster.

GENERAL INTRODUCTION

Parasites are significant sources of mortality in wild animal populations (Hudson et al. 2002, Moore & Wilson 2002). They seriously affect the health status of their hosts, mainly because fitness costs caused by parasites and associated diseases are high, they are ubiquitous, and most animals suffer from multiple infections simultaneously.

Since parasites play a prominent role in wildlife, it is of fundamental importance to assess parasitism and associated pathology in wild hosts. It is crucial to identify host-intrinsic and environmental factors that influence parasite fauna, prevalence and intensity of infections and the probability of developing pathology caused by these parasites (Nunn & Altizer 2006). Along with reproductive success and predation, infectious disease (including parasitism) is a major demographic and evolutionary driver of natural populations.

Studies on the parasites of marine mammals are important as they can provide data on the population ecology of their hosts, such as natural mortality factors (Perrin & Powers 1980, Raga et al. 1997), stock identity (Balbuena et al. 1995), distribution and diet (Dailey & Otto 1982), as well as social behaviour (Balbuena & Raga 1994). Under the assumption that different parasitic faunas indicate that the host populations are isolated or nearly so, parasites have been used as biological indicators for small odontocetes (Walker 1990, Aznar et al. 1995) and mysticetes (Dailey & Vogelbein 1991). Many studies have revealed the importance of host traits like diet and geographic range for the parasite fauna on individual, population and species level (Côté & Poulin 1995, Gregory et al. 1996, Morand & Poulin 1998, Arneberg 2002, Altizer et al. 2003, Nunn et al. 2003, Ezenwa 2004, Vitone et al. 2004), and recently, with the advent of DNA technology, the use of parasites as proxies for host genealogy and ecology has been discussed (Nieberding & Olivieri 2006).

This thesis aims to characterise the parasite fauna infecting harbour porpoises (*Phocoena phocoena*, Linnaeus 1758) and harbour seals (*Phoca vitulina*, Linnaeus 1758) in German waters. The objectives were

- (1) to qualify and quantify the occurrence and prevalence of the parasite species of porpoises and seals,
- (2) to assess the impact of parasites on the health status of their hosts,
- (3) to establish and use molecular tools to elucidate the ecology of nematodes parasitising the respiratory tract, and
- (4) to investigate the life cycle of the metastrongyloid lungworms.

SCIENTIFIC BACKGROUND

STUDY ANIMALS

Harbour porpoises are among the smallest cetacean species (1.6-1.8 m of length) and inhabit coastal or shelf waters of the northern hemisphere (Reijnders & Lankester 1990, Benke et al. 1998). They have a compact torpedo-shaped body of grey coloration and a small triangular shaped dorsal fin, homodont spate-shaped teeth but no distinct beak like dolphins. They belong to the suborder *Odontoceti* (toothed whales), and genetic and morphological investigations have indicated that harbour porpoises from the German North and Baltic Seas belong to three different subpopulations: southern and central North Sea, western Baltic (including Kattegat, inner Danish and German waters) and eastern Baltic (Tiedemann et al. 1996, IWC 2000, Kaschner 2001, Huggenberger & Benke 2002). Harbour porpoises occur predominantly solitary or in small groups, e.g. mother-calf pairs (Siebert et al. 2006a), and as fully aquatic animals they remain submerged most of the time. They reach sexual maturity at the age of three to four years (Benke et al. 1998, Bandomir et al. 1999, Lockyer & Kinze 2003) and after 10 months of gestation females give birth in June/July to one calf of 65 to 75 cm of length, shortly before mating again. Their lactation period lasts approximately 10 months but newborns begin to additionally feed on fish at approximately 5-6 months of age (Schulze 1996). Most adult females are both pregnant and lactating at the same time which contributes to their high energy demand during that time (Read & Hohn 1995, Read et al. 1997, Reeves et al. 2002). Harbour porpoises are opportunistic and benthic feeders and feed primarily on goby and cod in the North Sea (Gilles et al. 2008). In the Baltic Sea cod, herring and goby seem to be the most important prey (Koschinski 2002, Lockyer & Kinze 2003, Gilles et al. 2008). Harbour porpoises use echolocation for foraging, navigation and communication (Kamminga & Wiersma 1981, Au 1993, Akamatsu et al. 1994, Verfuß et al. 2005) and have a delicate sense of hearing. The animals are therefore sensitive to the effects of sound emissions, which can range from disturbance to impairment of finding prey, hearing loss and even death (Ketten 2000, Lucke et al. 2008). A first European survey for the estimation of harbour porpoise density in the North Sea and adjacent waters was conducted in 1994 (Hammond et al. 2002). A total of 341,366 harbour porpoises were estimated (95% CI: 260,000-449,000). A second survey was conducted in 2005 and resulted in an estimate of

385,617 animals (95% CI: 261,266-569,153), a comparable count but with a different distribution and area surveyed (SCANSII 2008).

Bycatch in fisheries represents a large threat for harbour porpoises. Each year several thousand animals are killed in fishing nets in the North and Baltic Seas (Vinther 1999, ASCOBANS 2000, Vinther & Larsen 2004). Chemical pollution (Jepson et al. 1999, Siebert et al. 1999), food depletion and noise pollution lead to degradation of this species' habitat (Reijnders 1992).

Particularly the eastern Baltic population is considered highly endangered. The population size is estimated to comprise only several hundred individuals (Berggren et al. 2003). Bycatch from fisheries exerts continuous pressure on the population (Skóra & Kuklik 2003). To address these issues, the 'Recovery plan for the Baltic harbour porpoise' (Jastarnia Plan) was adopted by the ASCOBANS (Agreement on the Conservation of Small Cetaceans of the Baltic and North Sea) member states to prevent its extinction. An overview of national and international agreements concerning harbour porpoises in European waters is given by Kaschner (2001) and Evans et al. (2003).

The **harbour seal** belongs to the suborder *Pinnipedia* (pinnipeds) and superfamily *Phocoidea* (seals). The harbour seal reaches a length of 1.75 m. Different subspecies are widely distributed throughout the Atlantic and Pacific coastal regions of the northern hemisphere (King 1983, Reijnders et al. 1997, Perrin et al. 2002). The harbour seals breeding on haul-out sites in the German Wadden Sea belong to a population inhabiting the south-eastern North Sea and they are rarely seen in the German Baltic Sea. The animals are of grey-brownish colour with dark spots and a typical round-shaped head. They have retained an amphibious lifestyle and are dependent on land for parturition, nursing and moulting. Although they remain in large colonies while on land, they are predominantly solitary when at sea (Herr et al. 2009). Harbour seals reproduce annually and give birth in the German Wadden Sea typically from May to July with a peak of 95% of births in June (Abt 2002). The offspring is nursed for about 4 weeks. Sandbanks and sometimes beaches are particularly used during the birth and lactation period as well as the annual moult. The latter takes place during July and August. Animals also use haul-out sites for resting during other times of the year, though to a lesser extent. Harbour seals travel long distances into the North Sea for feeding (Reijnders et al. 2005, Adelung et al. 2004). Harbour seals are opportunistic feeders that, in

the German North Sea, mainly prey on different flatfish species and other demersal fish, like gadoids (Gilles et al. 2008). They find prey primarily with the help of their vibrissae which are able to locate fish or water turbulences caused by fish movements (Dehnhardt et al. 1998, 2001). Harbour seals, like other pinnipeds, produce sounds in the air and underwater for communication (Schusterman & van Parijs 2003). Vocalisation plays an important role in maintaining contact between mother and offspring (Khan 2004).

The population of harbour seals (*Phoca vitulina*) in the Wadden Sea decreased until the mid-1970s. Anthropogenic impacts such as hunting (Reijnders 1983, Heide-Jørgensen & Härkönen 1988), chemical pollution (Reijnders 1986, Brouwer et al. 1989) and disturbance (Reijnders 1983, Thiel et al. 1992) were highly suspected to be responsible for the decline of the harbour seal population in the Wadden Sea. The management and conservation of harbour seals rose to new prominence under the Trilateral Wadden Sea Agreement. Coordinated aerial surveys allowed monitoring of the population size during the last 30 years (Abt 2002, Reijnders et al. 2005). The banning of hunting and the establishment of national parks in German waters led to the recovery of the population (Abt 2002, Reijnders et al. 2005). In 1988 the North Sea harbour seal population was struck by the first die-off caused by Phocine Distemper Virus (PDV) (Kennedy 1998). Over 18,000 harbour seals died, accounting for 55-65% of the population (Reijnders & Brasseur 2003). Anthropogenic activities could not be excluded as a contributing factor because immunological investigations revealed that the impairment of the immune response by chemical pollutants increased the susceptibility to viral diseases (De Swart et al. 1996, Ross et al. 1996). 14 years later, the seal population had recovered to five times the initial population size before the second seal die-off began spreading around the North Sea (Jensen et al. 2002). The seal population was reduced by the seal die-off from approximately 21,000 counted seals in the Wadden Sea in 2002 to 11,000 counted seals in 2003 (Reijnders et al. 2005) but has since recovered to 20,250 in 2008 (TSEG 2008).

PARASITISM IN MARINE MAMMALS

Developing adequate conservation plans for marine mammal populations that are currently threatened or endangered is a matter of public concern. Recent epizootic events resulting in mass mortalities, like the well documented PDV epidemic in the Wadden Sea which decimated the harbour seal population in 1988/89 and in 2002 (Härkönen et al. 2006), have

increased the awareness of decision-makers, but also of biologists. Virus (microparasites) causing epizootics are perhaps the most important parasite group in terms of regulating populations. Macroparasites, like endoparasitic nematodes, are known to seriously affect the health status of their hosts. However, also due to logistical and methodological restraints, still too little is known about the role of parasitism in the dynamics of marine mammal populations.

Methodology

Marine mammal parasites are closely linked to the aquatic environment of their hosts and there is usually little control over their sampling conditions – first, because it is difficult to gain access to these animals in the wild, but also due to ethical implications in working with them. Researchers often have to rely on unpredictable, occasional strandings or bycatches of marine mammals, which may result in low sample sizes and/or severe sampling biases. For instance, it may be difficult to obtain age-stratified data to study variation with host age, or small sample sizes may miss heavily infected hosts. Studies based on stranded animals might be unreliable because they are usually diseased and may not be representative of the whole population. Additionally, many aspects of the biology of marine mammal parasites are poorly studied, the life cycles of most species being incompletely known (Raga 1992, 1994). These shortcomings hamper the development of epidemiological models, which can help understand the behaviour and consequences of parasite infections.

Many different factors are assumed to shape parasite fauna in hosts at any stage of the actual infection: parasite encounter, transmission, parasite recruitment, colonisation, parasite reproduction and establishment. All of these parameters are difficult to measure in wild host populations, thus indirect surrogates are needed, e.g. (1) parasite intensity denotes the number of parasite individuals of a particular species per host (Margolis et al. 1982, Bush et al. 1997) whereas (2) parasite prevalence is the number of hosts infected with a particular parasite as a proportion of all hosts examined (Margolis et al. 1982, Bush et al. 1997). These terms are used as quantitative descriptors for parasite ecology in host populations. Prevalence, being based on presence/absence data is a robust metric.

As the host represents the well-defined habitat on which the parasite depends strongly for at least one stage of its life history, it is of interest to study host traits in search of ecological, behavioural and morphological correlates for parasite prevalence. Social interactions provide key opportunities for parasite spread, and parasites transmitted by social contacts

are expected to be more common in larger groups and in denser populations (Freeland 1976, Loehle 1995, Arneberg et al. 1998).

When stocks of host species are separated geographically in different environments, their respective parasite faunas will exhibit differences (Noble & Noble 1982). This is particularly true for helminths that generally require more than one host to complete their life history. The occurrence of a particular parasite in any geographic locality depends on the presence of a suitable definite host, suitable intermediate host(s) and complex biological factors which impart a strict interdependency on the organisms comprising the host-parasite complex.

Impacts of parasites on marine mammals

Parasites exert selective pressure on the evolutionary responses of hosts at the immunological and behavioural level, just as host behaviour and immune defenses may have evolved to reduce the spread and pathogenicity of parasites.

Anthropogenic activities, such as fisheries, gas and oil exploration, chemical industry and offshore wind farms in the North and Baltic Seas, are causing various ecological changes which affect harbour porpoises and harbour seals today. Although the immune system of these animals is designed to accommodate stressful situations to some extent, an imbalance can be created through additional impacts by chemical pollutants, prey depletion and continuous stress, such as human activities (see chapter I). An impairment or suppression of the immune system caused by cumulative effects may lead to higher parasitic burdens, the proliferation of infectious diseases and can result in the death of animals (De Swart et al. 1996, Ross et al. 1996).

However, parasites can have positive effects on their hosts and be responsible for the stimulation of the immune system. There is increasing evidence that helminth infections can protect the host against allergic pathologies ('hygiene hypothesis') in human hosts (Maizels 2005). Elevations of anti-inflammatory cytokines, such as interleukin-10, that occur during long-term helminth infections have been shown to be inversely correlated with allergies (Yazdanbakhsh et al. 2002). Parasites are often long-lived and inhabit immunocompetent hosts for prolonged periods. Consequently, it is not surprising that they should possess modulatory molecules that ameliorate host responses to enhance their own survival (Yazdanbakhsh et al. 2002). Parasitic infections are known to induce protective immunity in terrestrial mammals (Pflugfelder 1977) and this may be the reason for significantly higher

infection rates with lungworms in young age classes of seals compared to adults (see chapter III).

Parasites can exert an important impact on population dynamics of their hosts in terms of reducing fecundity and/or survival of the host individuals (Scott & Dobson 1989, Hudson et al. 2002) but they are nevertheless a common finding in wild animals (see chapter I). It is difficult both to determine which parasitic load should be considered 'normal' and to establish a threshold at which parasites and associated lesions are becoming pathogenic.

COEVOLUTION OF MARINE MAMMALS AND PARASITES

The evolution of marine mammals in the ocean led to their ecological and evolutionary isolation. The adaptation of cetaceans and pinnipeds to aquatic life, on the one hand, allowed the development of new relationships to marine invertebrates and, on the other hand, enabled some fauna associated with terrestrial ancestors to persist and undergo a drastic adaptation to marine life (Anderson 1984).

Most parasites of **cetaceans** represent little diverging relicts of the fauna that originally parasitised the terrestrial ancestors of the cetaceans. Especially the lungworm family Pseudaliidae (Metastrongyloidea) represents a phylogenetically very old group of parasites that has disappeared in terrestrial mammals and persisted in the aquatic environment through the ecological isolation of their marine mammal hosts. Nowadays, all pseudaliid genera except one parasitise toothed whales (Anderson 1982).

The connection between crustaceans and cetaceans is younger on an evolutionary timescale. The complex of organisms that are locomotive with a cetacean has been described as a migrating biocenosis (Tomilin 1967). The respective parts of this community are characterised by many different relationships, like commensalism and ectoparasitism and between distinct fauna, e.g. algae and crustaceans, and they are characterised by varying degrees of specialisation. Some commensals may be found also on fish or turtles and even on inanimate items like drifting wood. Other ectoparasitic crustaceans, like amphipods from the cyamid family that also infest harbour porpoises (see chapter II), are highly host-specific and can only survive and reproduce on cetaceans (Rowntree 1996).

The amphibian life style of **pinnipeds** has influenced their parasite fauna so that some parasite groups still remain dependent on land to complete their life cycle. The transmission of seal lice parasitising harbour seals occurs on land, mostly from mother to pup, sometimes from pup to pup and seldom from adult to adult (Kim 1975).

Most multicellular parasites of seals belong to the insects, mites and helminths and they were probably derived from their terrestrial ancestors. The several species of lice (Anoplura) that parasitise seals are among the few insects that have adapted to the marine environment. They are arthropods that were closely associated with the terrestrial relatives of seals and have further specialised and adapted to marine carnivores. A hypothesis on the phylogeny of otariidae was formulated based on seal lice (Delyamure et al. 1982).

The fact that otariids and phocoenids are both parasitised by nematodes belonging to the family Filaroididae indicates that they may have been derived from terrestrial carnivores. The genus *Otostrongylus* (Crenosomatidae) occurs in Phocidae and close relatives of this nematode genus occur in mustelids and ursids. The investigation of pinniped parasite fauna has yielded insights not only into phylogeny and biogeography of pinnipeds but also given information about relict species.

Parasite-host relations can be so old that conclusions about common ancestors may be drawn from the occurrence of the same parasite species in modern pinnipeds (Delyamure et al. 1982).

Helminths may also occur in marine mammals as a consequence of 'host-catching'. Parasites normally infecting other vertebrates like fish or birds acquire new hosts like cetaceans or pinnipeds via the food web because they prey on the same items and may be used as final hosts as well. This is the case in tetrabothriid cestodes that usually infect seabirds through intermediate fish hosts and have adapted to marine mammals because of their similar diet (Hoberg 1989, Hoberg & Adams 1992).

A special trait of all parasitic worms of marine mammals is their remarkable ability to adapt to the marine environment and develop a heterogeneous or indirect life cycle as well as recruit new intermediate and paratenic hosts (see chapter IV).

LIFE CYCLE & ECOLOGY OF PARASITES

A main characteristic of parasites is their ability to infect hosts, causing tissue damage and consequently disease. The pathways and mechanisms used to infect hosts can vary greatly among different parasite groups and species.

Therefore, parasitism cannot be investigated without considering parasite ecology in terms of life cycle, transmission mode, host specificity as well as host and parasite habitat characteristics. Since, in the ecological sense, the term 'parasite' includes a wide range of organisms, parasite types diverge in their mode of replication and transmission, generation times, elicited immune responses and diseases (Hudson et al. 2002).

Host habitat, social organisation and mating system influence not only parasite diversity and prevalence but may also determine the fitness advantages of different transmission strategies to parasites (Altizer et al 2003).

In order to understand host-parasite interactions, knowledge of the parasites' life cycle is crucial. A direct life cycle (homoxenous parasites) means that transmission occurs within individuals of one host species where adult parasites reproduce sexually and release eggs/propagules. An indirect life cycle (heteroxenous parasites) requires at least one other species as intermediate host where asexual reproduction of the parasite can take place (Bush et al. 2001, Eckert et al. 2005). The earliest nematode parasites of vertebrates probably had direct life cycles without intermediate hosts (monoxeny). The tendency to isolate the free-living stages from the rigours of the external environment (known as 'seclusion') eventually gave rise to heteroxeny or the use of intermediate hosts in which development to the infective stage took place (Anderson 1996). That is probably why, in the marine environment, monoxenous life cycles are rare. The evolution of transport hosts (paratenesis) probably adapted heteroxeny to the changing food habits of the host during the radiation of the vertebrates. With the appearance of heteroxeny and paratenesis in terrestrial vertebrate parasites, opportunities for transfer to the aquatic ecosystem appeared (Anderson 1996). However, new parasites could not evolve from free-living forms in aquatic habitats. Thus, the nematode fauna in marine mammals is restricted, lacks the diversity found in terrestrial vertebrates and retains systematic affinities to forms in terrestrial hosts from which they have evolved (see chapter IV). Food web context has been hypothesised to have an influence on transmission rates of some parasite species in aquatic systems (Curtis 1984, Marcogliese 2002). The transmission routes of many parasitic

helminths follow closely the trophic relationships among their successive hosts and are thus embedded in food webs in a way that may influence energy flow and the structure of the web (Thompson et al. 2005). This is the case in several fish species that serve as intermediate or paratenic hosts of some stomach parasites of piscivorous birds and mammals. For example, ascarid nematodes such as *Pseudoterranova decipiens* (of seals) and *Anisakis simplex* (of toothed whales) can have sizeable third-stage larvae in the intestine and muscle of marine fishes, which are infective to the final marine mammal host. They are of importance also because they are pathogenic for humans if ingested together with undercooked fish (Anisakiasis).

While reasonable knowledge is available for many parasites affecting terrestrial mammals, information on potential intermediate hosts or life cycles in the marine environment remains scarce.

EVOLUTIONARY HISTORY OF NEMATODA (METASTRONGYLOIDEA)

The phylum nematoda represents a diverse group of organisms, ranging from species that are free-living in soil or water to those which are parasites of plants or animals. They are assumed to be of ancient origin, emerging possibly in the Paleozoic or Pre-Cambrian Eras (Inglis 1965, Maggenti 1983, Bird 1986). The evolutionary relationships of major groups within this phylum are difficult to define because of limited fossil records, a lack of phylogenetically-informative morphological characters and an unstable classification at the ordinal and higher levels (Inglis 1983, Maggenti 1983, Bird 1986). Molecular techniques, such as DNA sequencing, provide an alternative source of characters to test hypotheses relating to the origins of different groups within the phylum Nematoda. The bursate nematodes (order Strongylida) represent one of the major radiations of parasitic nematodes in animals, yet the phylogenetic relationships of some of the superfamilies and families based on morphological data are speculative (Durette-Desset et al. 1994). The superfamily Metastrongyloidea that consists of about 180 species, classified into about 45 genera and 7 families, is confined to mammals (Anderson 1982).

The Metastrongyloidea consist of strongyloid nematodes that are grouped together on the basis of site specificity of adults in mammals (primarily in the respiratory system), a reduced copulatory bursa and heteroxenous life cycles (Anderson 1978). Included in the superfamily are several species that elicit pathology in the bronchi, bronchioles and lungs of their hosts

(Testi & Pilleri 1969, Stockdale 1976, Bolt et al. 1994, Baker et al. 1995). Although adults of most metastrongyloid taxa are parasites of the respiratory system, other predilection sites have evolved in some metastrongyloid taxa, including the central nervous system and musculature of cervids (elaphostrongyline nematodes; Protostrongylidae), frontal sinuses of mustelids (Skrjabinstrongylidae) and the circulatory systems represented by various species in the Angiostrongylidae and Pseudaliidae (Skrjabin et al. 1952, Anderson 2000). Most species for which life cycles have been demonstrated use gastropods as intermediate hosts. However, non-gastropod intermediate hosts also exist, such as fish intermediate hosts in the life cycle of *Parafilaroides decorus* in sea lions (Dailey 1970) and earthworms in the life cycle of *Metastrongylus* spp. in suids (Anderson 2000). Thus, a wide variety of predilection sites, mammalian hosts and life cycle strategies are known among the Metastrongyloidea. Despite this diversity, few attempts have been made to study phylogeny of the Metastrongyloidea. Traditional evolutionary hypotheses for this group were based on limited information, speculative views on the phylogeny of nematodes based on assumptions of host-parasite co-evolution and different interpretations of the relative significance of anatomical features of the nematodes (Skrjabin 1941, 1942, Dougherty 1949, Delyamure 1955). Durette-Desset et al. (1994) summarised the phylogeny of the metastrongyloids as a process involving life cycle modifications as a dominant evolutionary phenomenon, coupled with the extensive use of intermediate and paratenic hosts and the invasion of numerous extraintestinal sites. Advances in phylogenetic reconstruction, including the use of molecular data, have made it possible to study the phylogeny of nematodes using methods that can complement morphological characters (see chapter IV).

MOLECULAR METHODS

The identification of parasitic strongyloid nematodes is usually based on morphological criteria. Particularly in noninvasive studies on wild host populations, if the determination of parasite species is based on morphological features, the number of encountered parasite morphospecies might not reflect the true number of parasite species (or what at that time is considered a 'species'). Nowadays, a number of new biochemical and molecular techniques enable the molecular characterisation of different species and demonstrate the existence of morphologically similar species, indicating that morphology alone may be insufficient for the delineation of species. This is particularly the case for the eggs and larvae, and sometimes

the adults of one sex, where no diagnostic morphological features exist to determine which species they represent (Chilton et al. 1992, 1993, 1995; Beveridge et al. 1994). It has been shown that the second internal transcribed spacer (ITS-2) of rDNA provides genetic markers for species identification (Campbell et al. 1995, Hoste et al. 1995, Stevenson et al. 1996). This has been demonstrated by comparing the sequences of morphologically well-defined species (Hoste et al. 1995) or by the concordance of data derived from multi-locus enzyme electrophoresis and DNA sequencing (Chilton et al. 1995). Thus, ITS-2 sequences can provide unequivocal species markers independent of the presence or absence of reliable morphological characters.

DNA technology has had a major impact in many areas of parasitology, including the identification and systematics of parasites, the diagnosis of infections, the epidemiology of parasites, the analysis of population genetic structures, gene expression and organisation, the study of drug resistance and vaccine development. In particular, the advent of the polymerase chain reaction (PCR) (Saiki et al. 1985, Mullis et al. 1986) has revolutionised parasitological research and has found broad applicability, mainly because its sensitivity permits the amplification of genes or gene fragments from minute amounts of parasite material. This is of particular relevance because it is often not possible to obtain or isolate sufficient amounts of material from some parasites and their different life-cycle stages for conventional analyses (see chapter IV).

THESIS OUTLINE

This thesis consists of four chapters, each representing studies on different aspects of harbour porpoise and harbour seal parasites and their ecology. Three studies were published in peer-reviewed journals and the fourth chapter is submitted for publication to a peer-reviewed journal. The published studies are identical to their offprint versions in style, wording and in the format of literature citations in the text and reference list. The lay-out and position of the figures were adapted to the lay-out of the thesis. The references cited in each study are listed in each chapter, while the references for the introduction and synthesis are listed at the end of the thesis. The individual referencing leads to a duplication of some references throughout the thesis.

CHAPTER I

The occurrence and prevalence of the parasite fauna of harbour porpoises were investigated in this first detailed study on parasites of porpoises from German waters. I identified seven parasite species, including a new species (*Halocercus invaginatus*) that was recorded for the first time in German waters. Further, I correlated severity of parasite infections to pathological lesions in infected organs. To assess the impact of parasites on the health status of porpoises, I compared parasitic infection in bycaught and stranded porpoises and in animals from different geographic origins.

CHAPTER II

A rare finding of an ectoparasitic crustacean on a stranded harbour porpoise off Helgoland was described in this manuscript. The study contains the first record of the species *Isocyamus delphinii* (Cyamidae; Amphipoda) from German waters. During a detailed necropsy I preserved all parasites, documented the parasite community as well as the associated pathology and investigated the cause of death of the harbour porpoise. I linked the parasite species to oceanographic features of the North Sea and debated likely causes for the unusual occurrence of whale lice on a porpoise, taking into account host ecology and necropsy findings.

CHAPTER III

Parasitism in seals from the German North Sea between the two Phocine Distemper Virus (PDV) outbreaks 1988/89 and 2002 was investigated in this study. I placed special emphasis on the nematodes parasitising the respiratory tract and lesions associated with parasitic infection in the lungs and digestive tract. For the first time parasitism in PDV negative seals from the Wadden Sea were examined in detail, and I compared my results with findings from PDV-positive seals. Further, I correlated lungworm infection in harbour seals with their age.

CHAPTER IV

In the last manuscript, I elucidated the life cycle of six lungworm species infecting porpoises and seals. Using molecular methods I characterised the different nematode species by their species-specific ITS-2 (rDNA) sequences and established a phylogeny of these

metastrongyloid nematodes. Additionally, I used the sequence data and established an *in-situ* hybridisation to screen possible intermediate hosts for larval lungworm stages. This is the first study that identified vertebrate intermediate hosts for three lungworm species of porpoises and seals from the North Sea in the wild and by experimental infections. Three fish species, typical prey items of porpoises and seals, were tested for their function as suitable intermediate hosts.

CHAPTER I: MACROPARASITES IN STRANDED AND BYCAUGHT HARBOUR PORPOISES FROM GERMAN AND NORWEGIAN WATERS

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ABSTRACT

Parasitological investigations were carried out on harbour porpoises *Phocoena phocoena* originating from 3 different areas: the German North Sea (28), the German Baltic (18) and Norwegian waters (22). The individuals were bycaught in gill-nets or found stranded during the period 1997 to 2000. A total of 7 species of parasites was identified from the investigated organs. These originated mainly from the respiratory tract and comprised 1 ascaridoid nematode (*Anisakis simplex*), 4 pseudaliid nematodes (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*) and 2 trematodes (*Campula oblonga*, *Pholeter gastrophilus*). This is the first geographic record of *H. invaginatus* from German waters. Differences were found in the severity of the parasitic infection between stranded and bycaught animals as well as between porpoises from different areas. A correlation was shown between parasitic infection and observed lesions.

INTRODUCTION

Assessments of the health status of porpoises *Phocoena phocoena* in German waters have been undertaken since 1990. Special attention has been paid to parasites as major agents of infection. Parasitic infections have been found in the respiratory and alimentary tract, heart, liver, pancreas and auditory/cranial sinuses (Raga 1994, Siebert et al. 2001). Infections with lung worms are commonly associated with secondary bacterial infections (Balbuena et al. 1993, Kirkwood et al. 1997, Jepson et al. 2000, Siebert et al. 2002) and are known to seriously affect the health of marine mammals. Heavy burdens of lung worms cause obstruction of the respiratory tract, impede diving ability and can lead to death (Clausen 1985, Kirkwood et al. 1997). It remains unclear why porpoises from Greenlandic waters exhibit lower levels of parasitic infection and suffer less from lesions due to parasites than porpoises from German waters (Wünschmann et al. 2001). It has been suggested that the bioaccumulation of pollutants (PCBs, DDTs etc.) caused by environmental deterioration could be responsible for the impairment of immune responses to pathogens such as bacteria, fungi or parasites (Aguilar & Borrell 1995, Jepson et al. 1999, Siebert et al. 1999). In this study special attention was paid to the respiratory tract and to pathological lesions caused by parasites. The prevalence and abundance of parasite species in hosts stranded and bycaught and originating from different geographical areas were compared and correlated with bronchopneumonia and gastritis.

MATERIALS AND METHODS

Samples included in this investigation originated from dead harbour porpoises collected along the coasts of Schleswig-Holstein, Germany, between 1997 and 2000. All porpoises from the German North Sea (28) were found stranded, while 13 (72%) of the individuals from the German Baltic (18) were bycaught. Samples from Norwegian waters (22) originated from porpoises which were bycaught in commercial fishery gill-nets during 2000. The majority of the animals were stored at -20°C until necropsy, and some were dissected directly. Necropsies were performed according to international guidelines; all organs were examined macroscopically and histologically (Siebert et al. 2001). Furthermore, microbiological, serological and virological investigations were carried out on the porpoises. Parasites were collected during necropsies, preserved in 70% ethanol and identified

microscopically after preparation in lactophenol according to the scientific literature (Baylis 1932, Schmidt-Ries 1940, Yamaguti 1958, 1961, Arnold & Gaskin 1974). The level of parasitic infection was determined (during necropsy) semiquantitatively as severe, moderate or mild as described by Siebert et al. (2001). Age was determined according to Lockyer (1995) and porpoises were divided into ageclasses according to Siebert et al. (2001): ≤ 0.5 yr (neonates and calves); 0.5 to 4 yr (subadults); >4 yr (adults). Non-parametric tests were chosen according to the rationales indicated in Siegel & Castellan (1988). Exact tests were calculated when small samples required their use (Mundry & Fischer 1998), and 2-tailed p-values are indicated throughout. Fisher's exact tests and Mann-Whitney *U*-tests were calculated using SPSS 11.5.0, and Spearman correlations and corresponding p-values using software written by R. Mundry, Forschungs- und Technologiezentrum Westkueste, Hafentoern, Germany. Because the data frequently included tied observations, p-values were derived using a randomisation procedure.

RESULTS AND DISCUSSION

Seven species of parasites were identified from the samples investigated. Five species were nematodes: 1 ascaridoid nematode (*Anisakis simplex*) from the stomach and 4 pseudaliid nematodes (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*) from the respiratory tract and the cranial sinuses. In addition, 2 trematodes (*Campula oblonga*, *Pholeter gastrophilus*) were found. *P. gastrophilus* was found in the duodenal ampulla (fourth stomach compartment) and *C. oblonga* in the liver and pancreas.

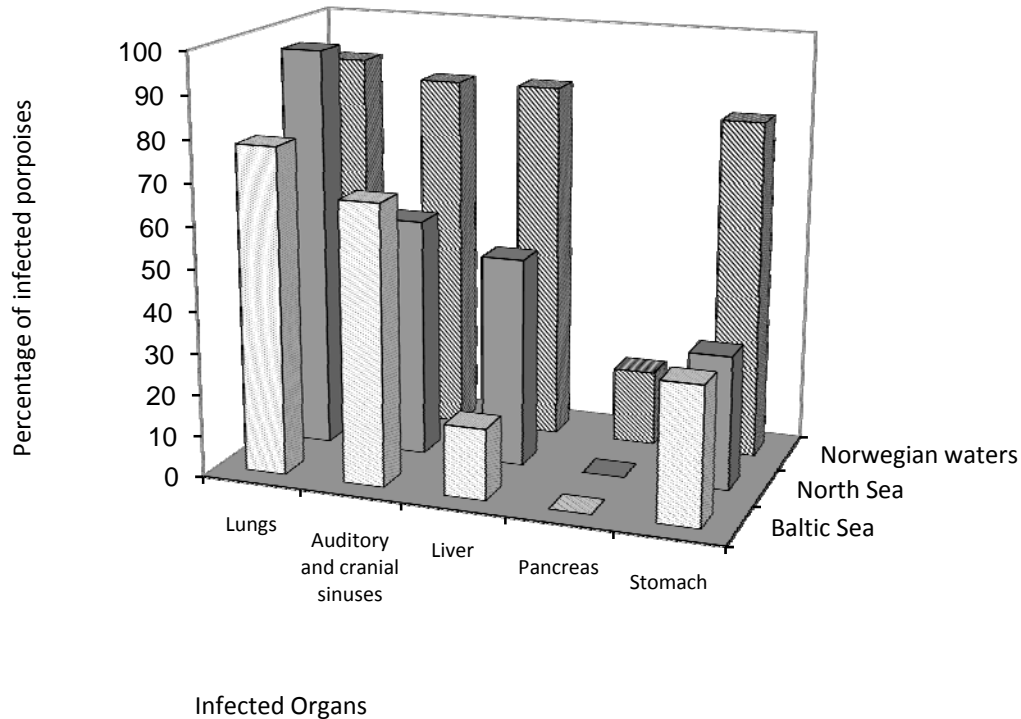


Fig. 1. *Phocoena phocoena*. Distribution of parasitic infection in the organs of porpoises in German (North Sea and Baltic Sea) and Norwegian waters

Parasites from the respiratory tract

Pseudalius inflexus, *Torynurus convolutus*, and *Halocercus invaginatus* were found in the lungs of porpoises from all areas, this being the first geographic record of *H. invaginatus* from the German North and Baltic Seas. Parasitic infection in the respiratory tract and in the ears and cranial sinuses was prevalent in most porpoises (90%) (Fig. 1). Nematode infection in the lungs of porpoises was often of mixed species and associated with bronchopneumonia. When pooling across all regions, age-classes, and bycaught and stranded individuals, there was a highly significant positive correlation between parasitic infection and bronchopneumonia ($r_s = 0.487$, $n = 68$, $p < 0.001$). *T. convolutus* was found in the nasal sinuses of 2 porpoises. Regarding the severity of parasitic infection in the respiratory tract of porpoises from different areas, animals from the Baltic Sea and Norwegian waters were less affected and did not exhibit severe levels of parasitic infection (Fig. 2). However, it has to be taken into account that all Norwegian porpoises and 13 (72%) Baltic Sea porpoises originated from bycatch, whereas all porpoises from the North Sea were stranded. As regards lung worm infection in bycaught and stranded porpoises, bycaught porpoises were less affected by parasites and associated bronchopneumonia (parasitic

infection: $n = 35$ bycaught, $n = 33$ stranded, $U = 295$, $p < 0.001$; bronchopneumonia: $n = 34$ bycaught, $n = 29$ stranded, $U = 256$, $p = 0.001$) (Fig. 3). In stranded individuals bronchopneumonia was found to be a common cause of death (Baker & Martin 1992, Siebert et al. 2001). Comparing bycaught porpoises from the Baltic Sea and Norwegian waters, individuals from the Baltic were generally less affected by both parasitic infection and bronchopneumonia than Norwegian porpoises, although only prevalence and intensity of bronchopneumonia differed significantly (parasitic infection: $n = 13$ Baltic, $n = 22$ Norwegian, $U = 98$, $p = 0.07$; bronchopneumonia: $n = 12$ Baltic, $n = 22$ Norwegian, $U = 75$, $p = 0.03$). It remains uncertain whether this is due to differences in the immune response of the hosts from different areas or whether diet and intermediate hosts differ in these regions. Obviously, bycaught individuals represent an overview of the population while stranded porpoises might have died because of illness. Two porpoise calves from the North and Baltic Seas examined in this study exhibited lung infection, which was determined in histology. Subadults predominantly showed mild or no infection, while all adults were infected, although severe infection was rare. These findings, which indicate a prenatal infection as suggested by Dailey et al. (1991), or an infection through lactation, are supported by Gibson et al. (1998), who found lung worms on histological slides of the lungs of newborn porpoises. Parasitic infection and associated bronchopneumonia tended to appear more severely in adults than in subadults (Mann-Whitney U -test; parasitic infection: $n = 21$ adults, 45 subadults, $U = 353.5$, $p = 0.08$; bronchopneumonia: $n = 18$ adults, 43 subadults, $U = 280.5$, $p = 0.081$).

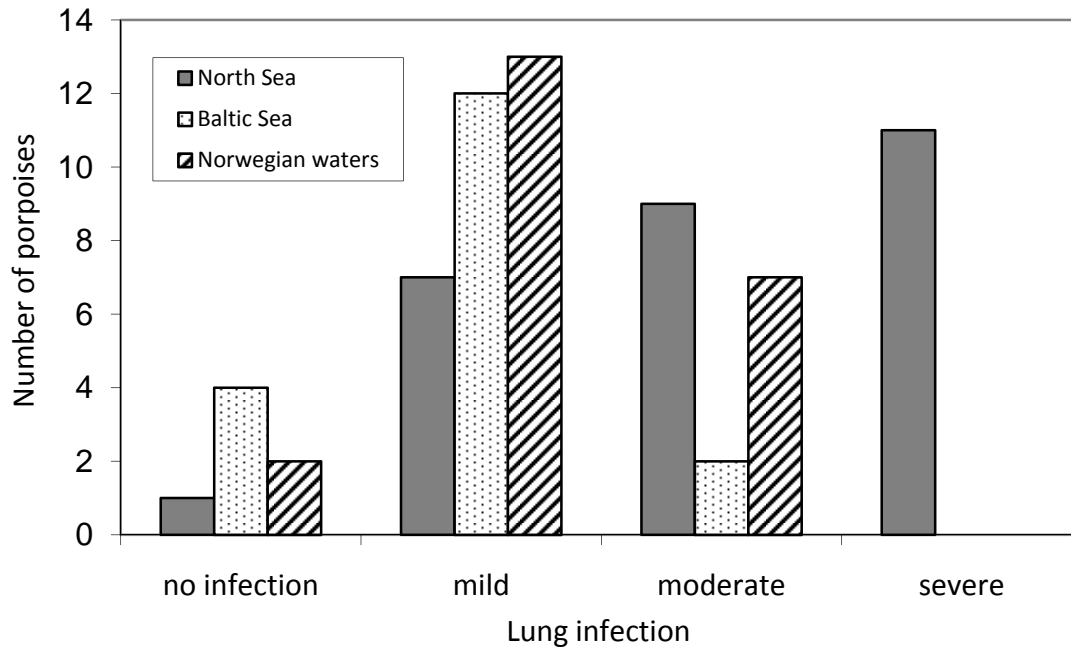


Fig. 2. *Phocoena phocoena*. Severity of infection by lung worms in the respiratory tract of porpoises from German and Norwegian waters. Distribution of parasitic infection in the organs of porpoises in German (North Sea and Baltic Sea) and Norwegian waters

Parasites of auditory/cranial sinuses

The nematode *Stenurus minor* infects auditory and cranial sinuses (Brosens et al. 1996) and was found in 47 porpoises from all 3 examined areas; some individuals were also found in the lungs. Contrary to the results of Faulkner et al. (1997), the infection of the porpoises examined in this work was not distributed evenly between the ears. Both stranded (62%) and bycaught (77%) porpoises were infected by *S. minor* and did not differ substantially in the severity of infection. In this study, porpoises infected with *Stenurus minor* exhibited no remarkable changes in the auditory sinuses and surrounding tissues. Other authors (Brosens et al. 1996, Siebert et al. 2001, Wünschmann et al. 2001) found no pathological changes associated with *S. minor*. Furthermore, the work of Faulkner et al. (1997), in which only bycaught porpoises in a good nutritional state were examined, supports the thesis that porpoises infected with *S. minor* are able to hunt effectively. Individuals found in samples from the lungs supposedly migrated there postmortem.

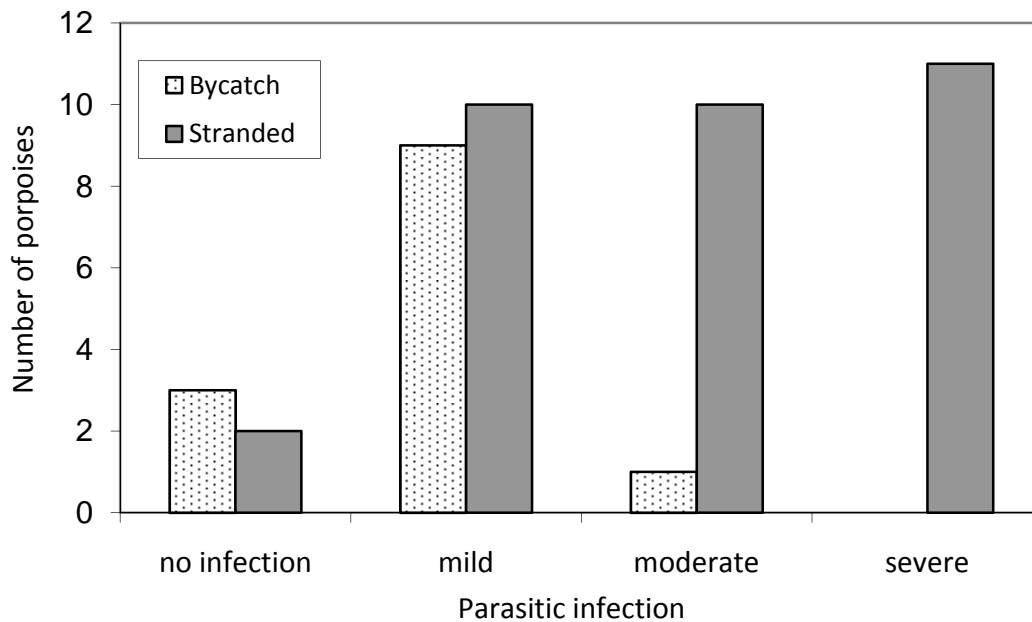


Fig. 3. *Phocoena phocoena*. Lung infection in bycaught and stranded porpoises from German waters

Parasites from liver and pancreas

The trematode *Campyla oblonga* was found in the liver of 36 porpoises from the North and Baltic Seas and Norwegian waters. No porpoise exhibited severe infection. When all harbour porpoises were divided into groups of individuals with and without infection of the liver, prevalence of parasitic infection in Norwegian porpoises was seen to be significantly higher ($n = 45$ subadults, $\chi^2 = 27.42$, $df = 2$, $p < 0.001$). In contrast to porpoises from the North and Baltic Seas, which showed no parasitic infection in the pancreas, 18% of those from Norwegian waters exhibited infections of this organ. Porpoises with trematodes in the liver and pancreas sometimes showed mild and moderate cholangitis, pericholangitis and periportal and periductular fibrosis. Siebert et al. (2001) reported *C. oblonga* in the liver and pancreas of porpoises from German waters and found similar lesions associated with the trematode. This probably does not impair porpoises seriously; however, the lesions can serve as an entrance for bacterial and fungal pathogens (Wünschmann et al. 1999).

Parasites from the stomach

Anisakis simplex was found in the first stomach compartment in 18 (80%) of the Norwegian porpoises, in 9 (32%) of the North Sea porpoises and in 6 (28%) of the Baltic Sea porpoises. Some individuals showed infections in the second compartment which were less marked: 7 (25%) Norwegian porpoises, and 1 porpoise each from the North (4%) and Baltic Seas (6%).

We assumed that our ascaridoid belonged to *A. simplex* sensu stricto, because it is the only species of the complex cited in the North Atlantic and Baltic Sea (Mattiucci et al. 1997).

Infection with *Anisakis simplex* in the first stomach compartment was often accompanied by ulcers. No difference in the severity of infection between subadults and adults was found (Mann-Whitney *U*-test; Baltic Sea bycaught porpoises: $n = 2$ subadults, $n = 11$ adults, $U = 9$, $p = 1$; Norwegian bycaught: $n = 3$ subadults, $n = 19$ subadults, $U = 24$, $p = 0.723$; Baltic Sea stranded: $n = 3$ subadults, $n = 2$ adults, $U = 2$, $p = 0.8$; North Sea stranded: $n = 13$ subadults, $n = 13$ subadults, $U = 81.5$, $p = 0.977$; no alpha adjustment applied), but bycaught porpoises from Norwegian waters were infected more often and more severely than bycaught individuals from the Baltic ($n = 13$ Baltic, $n = 22$ Norwegian, $U = 50$, $p < 0.01$). Lick (1991) also found markedly lower infection rates with *A. simplex* in Baltic Sea porpoises. The differences probably reflect a certain species composition in the diet of porpoises in different areas. Herreras et al. (1997) also found a strong local influence in the helminth community of the digestive tract in Danish porpoises. Regarding all porpoises, statistics showed that ulcers were associated with higher infection rates in the stomach (7 Mann-Whitney *U*-tests, calculated separately for 3 areas, 2 age-classes and 2 finding conditions: all except 1 (Norwegian porpoises) significant at $p < 0.05$). Smith (1989) associated ulcers in the forestomach of porpoises especially with *Anisakis simplex* larvae. *Pholeter gastrophilus* was found in 2 of the porpoises examined in the mucosa of the last stomach compartment. The trematode was accompanied by a mild granulomatous inflammation. Although it occurred rarely in individuals examined in this study, it appears to occur regularly in porpoises and, like *Campula oblonga*, to have no serious consequences for the host (Gibson et al. 1998). The genetic variation between Baltic and North Sea porpoises as well as Norwegian porpoises (Tolley et al. 2001) might influence their parasitisation to some extent. In addition, the diet composition of porpoises from the Baltic, North Sea and Norwegian waters could result in infection with different parasitic species. Differences in parasitic infection and associated lesions between porpoises from different regions might result from the level of pollution in their respective waters. Chronic exposure to contaminants such as PCBs and organochlorine compounds has been shown to be immunosuppressive in mammalian species (Jepson 1999).

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CHAPTER II: *ISOCYAMUS DELPHINII*: FIRST RECORD OF WHALE-LICE ON A HARBOUR PORPOISE (*PHOCOENA PHOCOENA*) FROM GERMAN WATERS

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SUMMARY

A case of whale-lice, *Isocyamus delphinii* (Crustacea: Amphipoda: Cyamidae), infestation is described in a dead stranded, juvenile female harbour porpoise (*Phocoena phocoena*) found on the coast of the island of Helgoland, in the North Sea (Germany). The emaciated animal showed multiple, diverse pronounced, partially scarred wounds of different sizes and shape with numerous small crustaceans. Furthermore, severe lungworm infection of the respiratory tract and moderate to severe pyogranulomatous pneumonia with marked interstitial fibrosis were found, as well as follicular hyperplasia of the spleen and lymph nodes. The ectoparasites are recorded for the first time in German waters and are an unusual finding on harbour porpoises in general. The whale-lice were associated with skin wounds, they showed a significant sexual dimorphism and their population was male biased.

INTRODUCTION

Infestation with whale-lice has been observed rarely in harbour porpoises (Stock 1973a, b) and *Isocyamus delphinii* (Crustacea: Amphipoda: Cyamidae) is seldom described from cold temperate waters, most records coming from oceanic or warm temperate waters (Rappé 1991). The last case from the North Sea was recorded in 1991 (Fransen & Smeenk 1991).

Heavy infestation by cyamids on cetaceans has been mainly described for slow moving baleen whales, but they are also known to infest odontocetes, although in lower prevalence and population densities (Rice 1963, Mizue & Murata 1951, Leung 1970b). The northernmost record of *I. delphinii* infestation on pilot whales (*Globicephala melas*) is from the Faroe Islands (Balbuena and others 1989, Raga and others 1988) and there are some reports of *I. delphinii* from Dutch waters, infesting a white-beaked dolphin (*Lagenorhynchus albirostris*) and several porpoises (Fransen & Smeenk 1991).

Cyamids, commonly known as whale-lice, are amphipod crustaceans that have a life-cycle without swimming stages and spend their entire life on a cetacean (Rowntree 1996). They attach to the skin of their host, preferably sheltered from currents at natural openings and wounds (Leung 1970a), and have developed adaptations like hooked claws and ventral spines to hold on to the cetacean's skin. They are thought to feed on sloughed skin of their cetacean host (Rowntree 1996). Transfer from one host to another can only occur through bodily contact between individuals during social interactions (Fransen & Smeenk 1991).

This is the first report of whale-lice, *Isocyamus delphinii* (Amphipoda), infestation on a harbour porpoise from German waters. The porpoise was necropsied as part of a research programme on monitoring the health status of small cetaceans of the German North and Baltic Sea.

MATERIALS AND METHODS

The harbour porpoise (*Phocoena phocoena*) was found stranded on the coast of the island of Helgoland, Germany, on the 1st of November 2005. It was delivered to the Research- and Technology Centre immediately and dissected on the same day. The necropsy was performed according to standard laboratory methods as used in Siebert and others (2001), all organs were examined macroscopically and histologically. Microbiological investigations

were also carried out. The macroscopic parasitic infection was determined semiquantitatively during necropsy as severe, moderate or mild as described by Siebert and others (2001) and was confirmed by histology. Parasites were collected during necropsy, preserved in 70% ethanol and endoparasites were identified microscopically according to Lehnert and others (2005). The ectoparasites were identified according to Leung (1967), Lincoln & Hurley (1974) and measured (from the anterior border of the head to the posterior border of the last segment of the pereon) using a binocular (Olympus SZH10 Research Stereo). Measurements were taken using a digital camera (Olympus Camedia) attached to the binocular with Olympus DP-Soft Version 3.2 software on a personal computer.

The nutritional state (body condition) of the porpoise was judged on the basis of blubber thickness and the state of muscles (Siebert and others 2001). The age was determined based on annual growth layers of the teeth (Lockyer 1995).

RESULTS

The examined porpoise was in good state of preservation, indicating that the animal had died recently. This is supported by the fact that some parasites were still alive. The female animal, measuring 102 cm and weighing 14 kg, was emaciated (blubber thickness between 8 and 13 mm) and estimated to be 1.5 years old (juvenile).

Pathological investigations revealed multiple wounds distributed over the whole body (Fig 1). The wounds were located ventrally and laterally and were of different shapes (round, longish or oblong with s-shape), different sizes (width between 0.5 cm to 1.5 cm, length from 3 cm to 6 cm) and depths were ranging from 0.5 cm up to 1.5 cm (through skin and blubber layer to the muscle). The margin of the lesions was either sharp or elevated by granulomatous tissue and bulging and sulcated. Some lesions were completely scarred. The scars were distributed dorsally and laterally. They were round, partly oval or longish, white pigmented, 0.5 cm in diameter and up to 10 cm long. Histopathological investigations determined multifocal, moderate, chronic, superficial, proliferative and histiocytic dermatitis, focal epidermic defects with slight perifocal purulent inflammation with slight fibrosis and focal severe, subacute, ulcerative dermatitis. The digestive tract was empty. The bronchial tree and pulmonary vessels were severely infected with nematodes. Investigations with a light microscope revealed multifocal moderate to severe pyogranulomatous pneumonia, diffuse mild interstitial lymphocytic pneumonia, mild lymphocytic bronchitis,

focal severe interstitial fibrosis, focal mild to moderate alveolarhistiocytosis and focal moderate alveolar emphysema. Spleen and lymph nodes displayed moderate to severe follicular hyperplasia. The glandular mucosa of the second stomach compartment was infiltrated slightly with lymphocytes.



Fig. 1: Live cyamids located on skin wound and scar on the right dorso-lateral body side of the harbour porpoise (scale bar = 1cm).

Numerous 0.5 cm crustaceans were located in and around the skin wounds (Fig. 1). 52 individuals were recovered from the skin and identified as *Isocyamus delphinii* (Cyamidae, Amphipoda). 30 (58%) were males, 18 (34%) females and four were (8%) juveniles, which could not be sexed. Several of the individuals clinging to the skin were still alive. Males (N=29) measured between 3208 μm and 4897 μm and females (N=18) between 3270 μm and 4034 μm in length. Males were significantly larger than females (Mann-Whitney U-test: U=159, Nfemales=18, Nmales=29, P=0.025) (Fig. 2) and the population consisted of more males. Juveniles (N=4) measured from 1139 μm to 1515 μm .

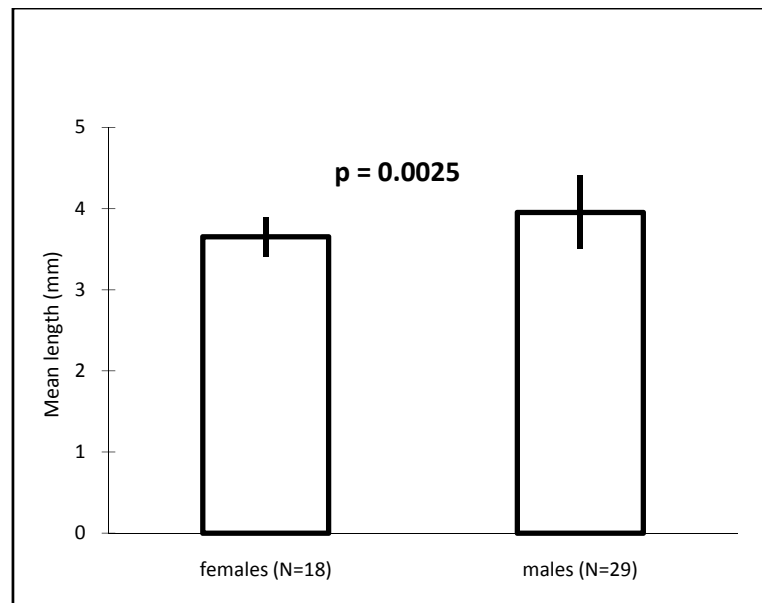


Fig. 2: Mean and standard deviation of the length of *Isocyamus delphinii*. Length difference is significant.

Three species of lungworms were identified from the respiratory tract, *Pseudalius inflexus*, *Torynurus convolutus* and *Halocercus invaginatus*, all belonging to the Pseudaliidae (Metastrongylida). Additionally both auditory sinuses and tympanic bullae demonstrated severe nematode infection with *Stenurus minor* (Metastrongylida, Pseudaliidae).

Microbiological investigations revealed mild or moderate growth of *Stenotrophomonas maltophilia* in liver, spleen, kidneys, lung, mesenteric and lung lymph nodes, intestine and skin. Furthermore a mild growth of *Staphylococcus aureus* was found in the skin, *Serratia* sp. in spleen, lung and lung lymph nodes, *Pseudomonas* sp. in spleen, kidneys and lung, *Erwinia* sp. in lung, lung lymph nodes and skin and *Acinetobacter* sp. was found in lung and lung lymph nodes.

DISCUSSION

The geographical distribution of *Isocyamus delphinii* is said to be approximately between 70°N and 60°W (Bowman 1955, Gruner 1975, Stock 1973a, 1977, Greenwood and others 1979, Raga and others 1983) so its occurrence seems well within its normal range. Rappé (1991) accounts the lack of *I. delphinii* records from the North Sea to this cyamids' preference of oceanic and warm temperate host species/populations and suggests that North Sea cetacean populations were free of cyamids until incursions of partly infested groups occurred in the 1960s. It is possible that infected animals from the North Sea are

originally oceanic stragglers or secondarily infected North Sea animals after intra- or interspecific contact with oceanic animals. So far *I. delphinii* has not been found on cetaceans from German North or Baltic Sea waters in ten years of intensive health monitoring programmes and neither on Danish, Swedish or Norwegian porpoises, suggesting that these porpoise populations are not a primary host species to this cyamid. The occurrence of *I. delphinii* in the North Sea has been discussed as being influenced by “oceanic years”, these being periods in which oceanic water masses are being pushed into the southern North Sea (Rappé 1991). Three cases of unidentified whale-lice on harbour porpoises have been reported from waters around Britain between 1992 and 2003 (pers. com. P. Jepson) and were also associated with severe skin lesions. The occurrence of *I. delphinii* could have been overlooked in previous strandings and necropsies of harbour porpoises and other small cetaceans, because whale-lice can be washed off after the death of an animal while it is drifting in the sea or be lost on the beach after stranding.

The cause of the skin lesions remains unclear. Mechanic alterations with secondary bacterial infection like lamprey marks (*Petromyzon marinus*) may produce these characteristic wounds, as described by Pike (1951) and Van Utrecht (1959). Lesions produced by social interactions with other porpoises or cetacean species might also be responsible. Whale-lice seem to be exhibiting some degree of site specificity on their hosts (Leung 1970a, 1976; Berzin & Vlasova 1982). The genital slit was by far the most preferred site by *I. delphinii* on pilot whales (*Globicephala melas*) off the Faroe Islands (Balbuena and Raga 1991). Balbuena and Raga (1991) suggest that higher prevalence and infection of *I. delphinii* on adult male pilot whales from the Faroe Islands resulted from sexual fights and inflicted fight marks, because heavy infections of *I. delphinii* observed by the authors always occurred on unhealed wounds of adult animals and it appeared that the number of whale-lice was positively correlated to the extent and severity of the lesions. The location around the genital slit proves useful for an ectoparasite that is dependent on social interactions among the host species for its distribution. Others report *I. delphinii* on the crease near the teeth of the pilot whale (Leung 1970a), or on the angle of the jaw (Greenwood and others 1979). In the presented case the female porpoise was neither infected in or around the genital slit nor around the mouth. *I. delphinii* was only associated with skin wounds, without primarily causing the skin wounds and infection. This indicates that the harbour porpoise may not be a normal host for *I. delphinii* and was only parasitized opportunistically because of its lesions. This fact also

suggests that *I. delphinii* is metabolically dependant on scar tissue (Sedlak-Weinstein 1992) and that only the crevices in the skin provided enough food and shelter to survive on this porpoise. Leung (1970a) reports sparse populations of cyamids on smaller, fast swimming and streamlined odontocetes.

If *I. delphinii* is not resident in the German North Sea porpoise population it is even more difficult to explain how it could spread to this single porpoise, taking into account that bodily contact between different dolphin/cetacean species in the wild appears to be uncommon (Fransen & Smeenk 1991). However, some species occur in mixed schools from time to time and, according to Fransen & Smeenk (1991), harbour porpoises (*Phocoena phocoena*) and pilot whales (*Globicephala melas*) as well as bottlenose dolphins (*Tursiops truncatus*) have been described to occur together. Epimeletic behaviour (caregiving behaviour) between species might be responsible for the distribution of cyamids (Fransen & Smeenk 1991).

Concerning the sex ratio of the cyamids on this porpoise (30 males (58%), 18 females (34%)), it is possible that not all cyamids were recovered. But in many cyamid species more males than females are available for mating at any time (Adams & Greenwood 1987) and Rowntree (1996) described a male biased sex ratio (70%) of *Cyamus boopis* (Amphipoda: Cyamidae) collected from a humpback whale. The presented data indicates that *I. delphinii* may have a male biased population. Cyamids do not synchronize moults and females mate immediately after moulting (Conlan 1991), so male competition has led to precopulatory mate guarding (males carrying females prior to molting) (Adams & Greenwood 1987). The significant difference in length of males and females is typical for cyamids, because males have to be able to carry females (Pouchet 1892).

The lesions in the lung were found to be the likely cause of death in this porpoise and the ectoparasites were not diagnosed to be a major health threat to the animal. The skin wounds' origin remains unclear, but the lice seemed to be closely associated and dependent on the wounds of this host. In this first and unusual occurrence of *I. delphinii* on a harbour porpoise from German waters the transmission was probably interspecific and skin wounds were likely the necessary means for the parasites to attach and remain on a porpoise.

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CHAPTER III: PARASITES IN HARBOUR SEALS (*PHOCA VITULINA*) FROM THE GERMAN WADDEN SEA BETWEEN TWO PHOCINE DISTEMPER VIRUS EPIDEMICS

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ABSTRACT

Parasites were collected from 107 harbour seals (*Phoca vitulina*) found on the coasts of Schleswig-Holstein, Germany, between 1997 and 2000. The prevalence of the parasites and their associated pathology were investigated. Eight species of parasites, primarily nematodes, were identified from the examined organs: two anisakid nematodes (*Pseudoterranova decipiens* (sensu lato), *Contracaecum osculatum* (sensu lato) from the stomach, *Otostrongylus circumlitis* (Crenosomatidae) and *Parafilaroides gymnurus* (Filaroididae) from the respiratory tract, one filarioid nematode (*Acanthocheilonema spirocauda*) from the heart, two acanthocephalans *Corynosoma strumosum* and *C. semerme* (Polymorphidae) from the intestine and an ectoparasite, *Echinophthirius horridus* (Anoplura, Insecta). Lungworm infection was the most prominent parasitological finding and secondary bacterial bronchopneumonia the most pathogenic lesion correlated with the parasites. Heavy nematode burdens in the respiratory tract were highly age-related and more frequent in young seals. A positive correlation was observed between high levels of pulmonary infection and severity of bronchopneumonia. The prevalence of lungworms in this study was higher than in seals that died during the 1988/89 Phocine Distemper Virus epidemic, and prevalence of acanthocephalans and heartworms had decreased compared to findings from the first die-off.

INTRODUCTION

The assessment of the health status of harbour seals (*Phoca vitulina*) in German waters has been undertaken since the seal die-off in 1988/1989. While searching for the cause of the epizootic in the Wadden Sea, detailed necropsies were performed on seals for the first time. It became obvious that a thorough knowledge of the parasitism in seals was needed to avoid misinterpretation of the necropsy findings. Later Phocine Distemper Virus (PDV) was identified as the cause of the first epizootic (Kennedy 1990) as well as for the second die-off in 2002 (Müller et al. 2002). During both epizootics the seal population in the Wadden Sea was significantly reduced by 40 - 60% (Härkönen et al. 2006). Since 1989 various research projects have been established to monitor the population dynamics and health status of seals. Special attention has been given to parasites as major agents of infection, but data on parasites of seals from the Wadden Sea originate mostly from seals that died during the 1988/89 PDV epidemic (Borgsteede et al. 1991, Claussen et al. 1991, Lunneryd 1992). Neither before nor after the first epizootic have systematic investigations of the parasitisation of seals in the North Sea been performed. Parasitic infections in seals have been found in the respiratory and alimentary tract, heart and on the fur (Raga 1992). Infections with lungworms are often associated with secondary bacterial infections and are known to seriously affect the health of young seals (Bergeron et al. 1997). High intensities of lung nematodes cause obstruction of the airways, impede diving ability and are a common cause of mortality (Dailey 1970). Gastrointestinal parasites may cause ulcers and inflammation and even perforation of the intestinal wall (Bergmann & Olsson 1985). In this study special attention was paid to lesions caused by parasites. The prevalence of parasite species (Bush et al. 1997) and level of infection in hosts from different age-classes were compared and correlated with bronchopneumonia and gastritis. The aims of this study were to determine the influence of parasites on the health status of seals unbiased by PDV and to analyse changes in the prevalence and level of infections over time.

METHODS

Samples originated from 107 harbour seals collected along the coasts of Schleswig-Holstein, Germany, between 1997 and 2000. 51 seals were found dead and 56 animals were killed

because of serious illness by a shot to the head by authorised national park rangers. The majority of the carcasses were stored at -20°C until necropsy; five were dissected while fresh. Necropsies were performed according to standard laboratory methods, as described in Siebert et al. (2001) for harbour porpoises, and were modified for seals. All organs were examined macroscopically and histologically. Microbiological, serological and virological investigations were also carried out (Siebert et al. unpublished data). Parasites were collected during necropsies, preserved in 70% ethanol and identified microscopically after preparation in lactophenol. The levels of parasite infection and pathological lesions were evaluated separately from each other and subjectively (Wünschmann et al. 2001) during necropsies and confirmed by histology. Macroscopic parasite infections were determined semiquantitatively: no=no parasites, mild=mild infection, moderate=moderate infection, severe=severe infection. Pathological lesions in organs infected with parasites were recorded and evaluated as no=no inflammation, no lesion, mild=focal mild inflammatory lesions, moderate=multifocal moderate inflammatory lesions or severe=multifocal severe inflammatory lesions (Siebert et al. 2001). Levels of parasitic infection (no, mild, moderate, severe) were correlated to pathological lesions (no, mild, moderate, severe) in infected organs. In 91 seals (85%) age was determined by tooth sectioning from cementum growth layers (Mansfield & Fisher 1960) and all seals were divided into age-classes: “age class 0” (born and deceased in the same year (approx. 6 months)); “age class 1” (seal born the previous year (up to 18 months)); and “age class 2” (animals >18 months). Non-parametric tests were chosen according to Siegel & Castellan (1988). Exact tests were calculated when small samples required their use (Mundry & Fischer 1998), and two-tailed P-values are indicated throughout. To interpret the results of multiple tests two approaches were used: firstly, a binomial test was used to test if the number of significant p-values corresponded to chance expectation (5%) (Cross & Chaffin 1982) and, secondly, Fisher’s Omnibus-test was applied (Haccou & Meelis 1994) when Mann Whitney U-tests (MW-U) were calculated using SPSS (11.0.1). For example shot vs. stranded and age class 0, 1 and 2 were factors by which the data were split (age class 2 shot animals were excluded due to small sample size). Tests were then calculated for each parameter (level of lungworm infection, level of pathological lesions in the lung, level of parasite infection in the heart, level of parasite infection in the stomach, level of pathological lesions in the stomach, level of parasite infection in the intestine, level of pathological lesions in the intestine, level of ectoparasite infection, level of

infection in pulmonary blood vessels) separately. Hence, five data sets (combinations of levels of factors) times nine parameters result in 45 MW-U tests and 45 P values. Parasite infections in the lung and in pulmonary blood vessels were grouped together after these tests (respiratory tract; see table 2), because they consisted of the same species. Generalized linear (GLM) and linear mixed effect (LME) models were performed using the statistical platform R (2.4.1). Parasite fauna and prevalence from the 1988/89 PDV epidemic published by Borgsteede et al. (1991), Claussen et al. (1991), Lynnerød (1992) and Strauss et al. (1991) was compared to data from this study.

Table 1: Age and sex distribution of 51 stranded and 56 shot seals.

Age class	Shot		Stranded		Total
	Male	Female	Male	Female	
0	17	11	7	8	43
1	14	11	13	7	45
2	2*	1*	10	6	19

*Shot seals from age class 2 were not included in some statistical tests due to small sample size.

RESULTS

Table 1 shows the age and sex distribution of shot and dead stranded seals. Eight species of parasites were identified from the samples collected, this being the first geographical record of *P. gymnurus* (Filaroididae; Metastrongyloidea) from Schleswig-Holstein (see **Table 2**). Five species were nematodes: two anisakid nematodes (*Pseudoterranova decipiens*, *Contracaecum osculatum*) from the stomach and two nematodes *Otostrongylus circumlitus* (Crenosomatidae) and *Parafilaroides gymnurus* (Filaroididae) from the respiratory tract. *Acanthocheilonema spirocauda* (Filarioidae) was found in the heart. In addition, two acanthocephalans (*Corynosoma strumosum*, *Corynosoma semerme*), belonging to the Polymorphidae, were found in the intestine and one ectoparasite (*Echinophthirius horridus*), Anoplura, was collected from the skin and fur (**Fig. 1; Table 2**). Neither intestinal nor liver flukes (Trematoda) nor cestodes were found in this study.

Table 2: Parasite species, location and prevalence in 51 stranded and 56 shot seals. ne = not estimated.

Parasites	Distribution	Prevalence % (n)		
		Stranded	Shot	Total
NEMATODA				
<i>Otostrongylus circumlitus</i>	bronchi, pulmonary blood vessels, heart	67 (34)	84 (47)	76 (81)
<i>Parafilaroides gymnurus</i>	bronchioles, lung tissue, nodules	ne *	ne *	ne *
<i>Pseudoterranova decipiens</i>	stomach	65 (33)	68 (38)	66 (71)
<i>Contraecum osculatum</i>	stomach	ne *	ne *	ne *
<i>Acanthocheilonema spirocauda</i>	right chamber, right atrium, heart	6 (3)	5 (3) 6 (6)	
ACANTHOCEPHALA				
<i>Corynosoma semerme</i>	intestine	2 (1)	0 (0)	1 (1)
<i>Corynosoma strumosum</i>	intestine	27 (14)	20 (11)	23 (25)
INSECTA				
<i>Echinophthirius horridus</i>	skin, fur	8 (4)	0 (0)	4 (4)

*was not attempted, because not all individuals were recovered from lungs and digestive tract

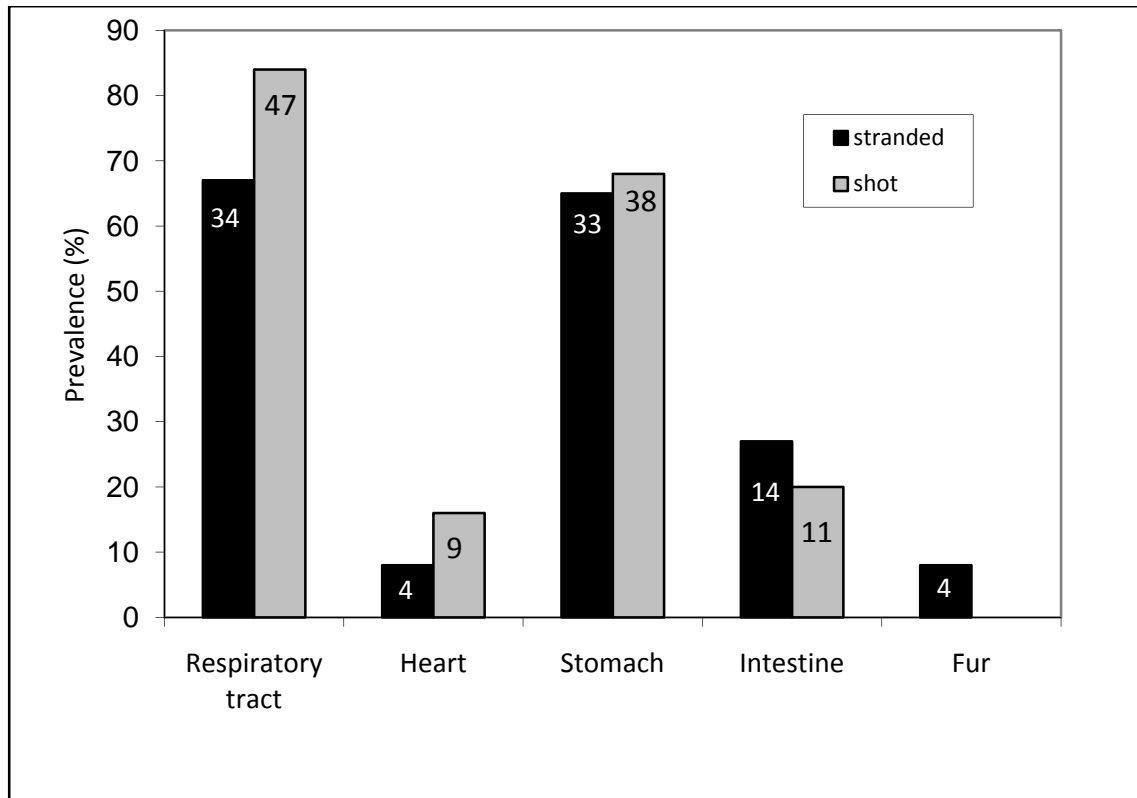


Fig. 1. *Phoca vitulina*. Prevalence of parasitic infections in respiratory tract, heart, stomach, intestine and fur in stranded (n=51) and shot (n=56) seals from the German Wadden Sea between 1997 and 2000. Numbers in columns are exact numbers of shot and stranded seals infected in different organs.

No differences in the levels of infection and associated pathological lesions were found between the sexes ($\chi^2=62.7$, $df=90$, $P=0.987$), and no differences in levels of infection and associated pathological lesions between stranded and shot seals were found ($\chi^2=57.7$, $df=108$, $P=0.999$) (Fig. 1; Table 3), so these groups were lumped for further statistical testing. With regard to the age distribution of stranded and shot seals, more age class 0 and age class 1 seals were shot due to serious illness ($\chi^2=13.6$, $df=2$, $P<0.01$). Older seals (age class 2) were mostly found dead (see Table 1).

The prevalence of parasites in seals from the Wadden Sea in the study period differed significantly (Pearson's Chi Square test, four values $P<<0.05$) from findings presented in previous studies conducted during the first PDV epidemic in 1988/89 (Table 5).

Table 3: Distribution of parasites and associated pathological findings between 51 stranded and 56 shot seals.

Findings	Prevalence in affected seals % (n)		
	Stranded	Shot	Total
Pulmonary nematodiasis	67 (34)	84 (47)	76 (81)
Suppurative, or suppurative-necrotizing bronchopneumonia	69 (35)	79 (44)	74 (79)
Cardiac luminal nematodiasis	8 (4)	16 (9)	12 (13)
Perforation of the pericard	2 (1)	0 (0)	1 (1)
Gastric nematodiasis	65 (33)	68 (38)	66 (71)
Gastritis	16 (8)	18 (10)	17 (18)
Intestinal parasitosis	27 (14)	20 (11)	23 (25)
Enteritis	18 (9)	13 (7)	15 (16)
Skin	8 (4)	0 (0)	4 (4)

Parasites from the respiratory tract

Eighty-one seals (76%) exhibited a parasitic infection of the respiratory tract with metastrongyloid nematodes (**Table 2**). Infections of *O. circumlitus* and *P. gymnurus* were often mixed and both species were grouped together for the correlation analysis. When pooling all age classes and sexes, both shot and stranded seals, a highly significant positive correlation ($P < 0.05$) was found between levels of lungworm infection and severity of bronchopneumonia (GLM: $r^2 = 0.41$). Bronchopneumonia, as a secondary bacterial infection associated with lungworms, was found to be the cause of death or severe illness leading to death in 56 seals (**Table 4**). The type of bronchopneumonia associated with lungworms varied between suppurative, suppurative-necrotizing and granulomatous bronchopneumonia with or without abscesses. Age class 0 ($n=43$) and age class 1 ($n=45$) seals showed a high prevalence (77% and 93%) of lungworm infections. As to the levels of parasitic infection in the respiratory tract of seals from different age classes, age class 0 and age class 1 seals were more severely infected than age class 2 seals (**Fig. 2**)

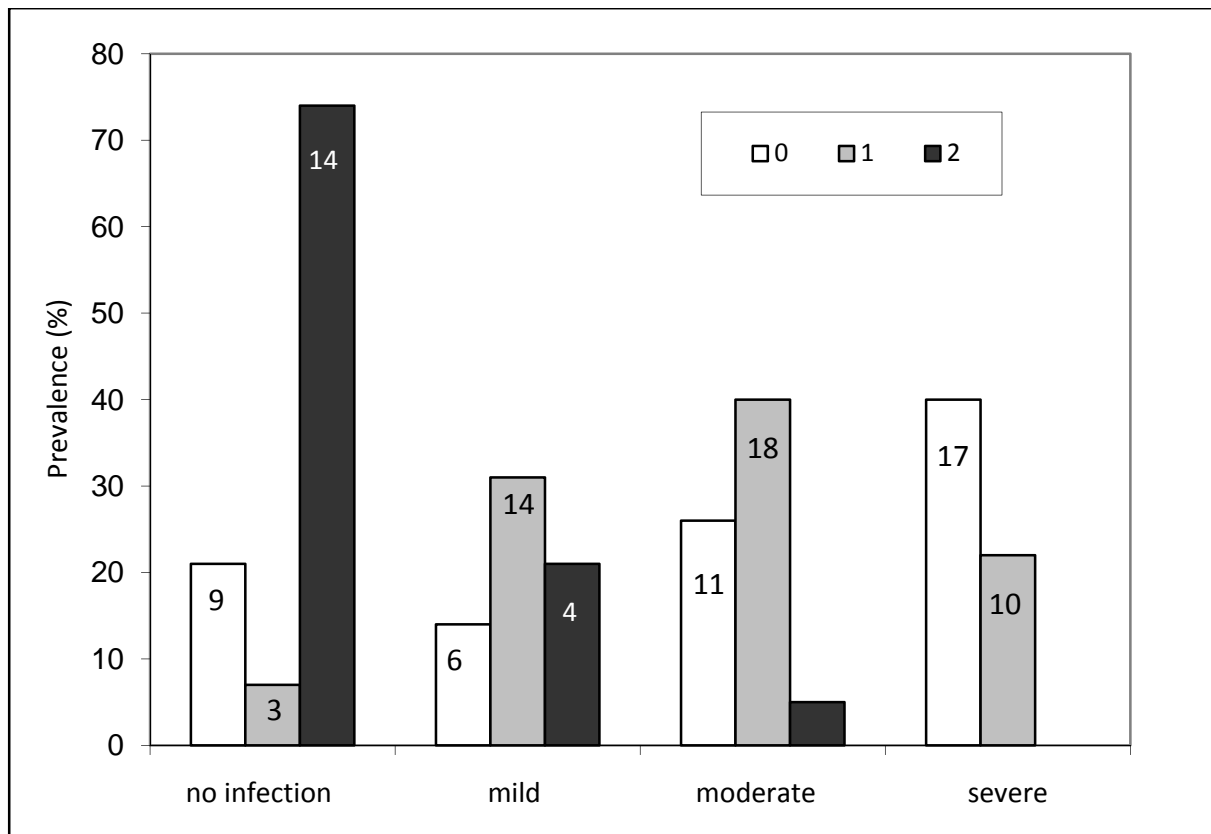


Fig. 2. *Phoca vitulina*. Prevalence of levels (no, mild, moderate, severe) of lungworm infection in harbour seals in age class 0, 1 and 2 from the German Wadden Sea between 1997 and 2000 (n=107). For definition of age classes see section M&M. Numbers in columns depict exact number of seals per age class.

(8 Kruskal-Wallis H tests, separately for each combination of sex and shot/stranded. $\chi^2=60.7$, df=16, $P<0.0001$). Only one seal from age class 2 suffered from a moderate lungworm infection, four animals from a mild infection. Considering the levels of pulmonary nematode infection and associated lesions in shot and stranded animals, no clear difference between shot and stranded animals was observed ($\chi^2=27.5$, df=24, $P=0.28$).

Parasites from the heart

Thirteen seals (12%) were infected with nematodes in the heart and pulmonary arteries: three animals were severely infected, two moderately and eight mildly. Six harboured *Acanthocheilonema spirocauda*, two of them being severely infected in the right chamber and atrium. One stranded seal was severely infected in the right atrium and had a perforation (2x3 mm) of the atrium and 15 ml of coagulated blood in the pericardium. This condition was determined to have been the cause of death. One animal was moderately infected with *A. spirocauda* in the right chamber, two showed a mild infection in the right

chamber and one of them additionally in the right atrium. Eight seals had mild *O. circumlitus* infections in the heart. All of those exhibited an additional infection with lungworms in the respiratory tract and/or pulmonary blood vessels. One seal had a mixed and mild infection of *A. spirocauda* and *O. circumlitus* in the heart. Animals infected with *A. spirocauda* were from age class 0 (n=2) or age class 1 (n=4).

Parasites from the stomach

Seventy-one seals (66%) were infected with stomach worms. The majority (55 individuals) had a mild infection. Thirteen animals were infected moderately, only three animals showed a severe infection. Stomach parasites were primarily *Pseudoterranova decipiens* (sensu lato), although some adult specimens of *Contracaecum osculatum* (sensu lato) were found. Eighteen seals in this study showed gastritis, 17 of them being infected with stomach worms. The character of gastritis in animals with stomach nematodes was in most cases diffuse catarrhalic and lymphocytic gastritis. Only one dead stranded female had a 1 cm ulcer in the stomach associated with stomach worms. A weak positive correlation between the level of stomach infection and gastritis was shown (all eight correlation coefficients positive, sign test < 0.008).

Parasites from the intestine

Twenty-five seals (23%) were infected with acanthocephalans in the intestine. *Corynosoma strumosum* was found in all seals, but *C. semerme* occurred only rarely (one animal) and in a mixed infection. 20 seals exhibited a mild infection, four a moderate and only one a severe infection. Sixteen of the animals studied had enteritis, but only eight of them were infected with acanthocephalans. The nature of the enteritis, when coinciding with acanthocephalans, was mostly a mild diffuse catarrhalic, lymphocytic plasmacellular enteritis. From 1997 to 2000 a significant increase in the level of the infection in the intestine was observed (LME: random factor: age group: $P(\text{time}) < 0.05$).

Ectoparasites

Four stranded seals were infected with *Echinophthirius horridus*, the seal louse, belonging to the Anoplura, Insecta. In two animals the infestation was severe, one seal was moderately infested and one animal mildly infested. None of the infested seals were infected with *Acanthocheilonema spirocauda*, for which *E. horridus* is believed to be an intermediate host (Geraci et al. 1981). One infected seal was age class 2 and three were age class 1. One severely infested seal (one year old) was found to have died of anaemia.

DISCUSSION

Infections with metastrongyloid lungworms were the most prominent parasitological finding in this study. Lungworm infection and associated secondary bacterial infections leading to bronchopneumonia were also major pathological findings leading to death (natural or by shooting) in more than half of the seals (**Table 4**). The lesions produced by nematodes can obviously enable the entry of bacteria or fungi (Wünschmann et al. 1999).

Table 4: Causes of death in 51 stranded seals and severe illness leading to death in 56 shot seals.

Fatal conditions	Prevalence in affected seals % (n)		
	Stranded	Shot	Total
Endoparasitosis	6 (3)	9 (5)	7 (8)
Bronchopneumonia	43 (22)	61 (34)	52 (56)
Intestinal dislocation	10 (5)	0 (0)	5 (5)
Dermatitis	2 (1)	4 (2)	3 (3)
Anemia	2 (1)	0 (0)	1 (1)
Perforation of atrium	2 (1)	0 (0)	1 (1)
Not diagnosed	22 (11)	9 (5)	15 (16)
Others*	10 (5)	9 (5)	18 (10)

*include single cases of: dystocia, trauma, keratitis, myositis, sepsis

Our data show that shot seals were diseased and eventually would have died shortly after from natural causes, if they had not been shot. The absence of flukes and cestodes in this study suggests a shift in either prey species or a change in immune status of seals in the Wadden Sea.

Lungworm infection in seals in this study was highly age-related; seals from age class 0 and 1 were especially affected by heavy burdens of nematodes in the respiratory tract. In contrast, the harbour porpoise (*Phocoena phocoena*) calving in the same habitat does not exhibit strong age-dependent lungworm infections (Lehnert et al. 2005), although lungworm species of harbour porpoises and harbour seals are quite closely related (Carreno & Nadler 2003) and the life cycles therefore may be similar (Lehnert et al. unpublished data). The differences might be due to the longer lactation period of porpoises of about eight months (Møhl-

Hansen 1954). Harbour seal pups, weaned after approximately four weeks (Ross et al. 1994), may have a suppressed or still not completely developed immune system (Lalancette et al. 2003), which could result in pups being more susceptible to infective larvae in prey items. We conclude that some uninfected pups from age class 0 were probably not infected because they died before they were weaned. Different feeding habits of the different age classes could also account for higher infection rates in young seals. The stimulation of immunity seems to be responsible for the statistically significant higher infection of lungworms in young age classes compared to adults (Claussen 1991). It is known that repeated infections with lungworms in cattle stimulate a highly protective immunity (Pflugfelder 1977).

Table 5: Prevalence % (n) of harbour seals infected with parasite species as examined in previous studies. x = not investigated.

Species	Borgsteede et al. (1991)	Strauss et al. (1991)	Claussen et al. (1991)	Lunneryd (1992) n=158; n=146	This study n=107
<i>Otostrongylus circumlitus</i>	6 (6)	x	26 (30)	0 (0)	76 (81)
<i>Acanthocheilonema spirocauda</i>	25 (23)	x	32 (37)	11 (18)	6 (6)
<i>Pseudoterranova decipiens</i>	59 (55)	x	88 (101)	x	66 (71)
<i>Corynosoma strumosum</i>	70 (66)	95 (104)	x	32 (46)	23 (25)
<i>Cryptocotyle lingua</i>	75 (70)	71 (78)	x	x	0 (0)
<i>Phagicola septentrionalis</i>	66 (62)	36 (39)	x	x	0 (0)
<i>Diphyllobothrium</i> sp.	9 (8)	32 (35)	x	0 (0)	0 (0)

The high prevalence of parasitic infection in the respiratory tract (76%) recorded in this study was not found during the 1988/89 epidemic by Claussen et al. (1991), who recorded a prevalence of 26% in seals from Lower Saxony and by Borgsteede et al. (1991), who only found a prevalence of 6% in waters around the Netherlands.

In this study *A. spirocauda* was predominantly found in young seals and did not impact their health, which supports the observation by Lunneryd (1992), who reported it mainly in young animals without affecting their condition. Compared to findings from the first PDV epidemic (25% prevalence found by Borgsteede et al. 1991 and 32% prevalence found by Claussen et al. 1991), the occurrence of *A. spirocauda* in seals from the Wadden Sea has decreased. *Otostrongylus circumlitus* presumably migrates to the heart via the bloodstream through pulmonary blood vessels or via a postmortem migration before the carcasses were frozen. The prevalence of parasitic infection in the stomach (66%) differed from that other authors found during the first PDV epidemic in 1988 (88% found by Claussen et al. (1991), 59% found by Borgsteede et al. (1991), 64-100% by Lunneryd (1992) and 36-98% found by Lick (1991). *P. decipiens* is clearly more abundant in harbour seals than *C. osculatum*, as found by Lick (1991).

The prevalence of acanthocephalans (23%) was low compared to 95% in seals from waters off Lower Saxony in 1988/89 (Strauss et al. 1991) and 70% in seals from the Dutch coast during 1988 (Borgsteede et al. 1991). An increasing trend in acanthocephalan infection levels was found during the study period. Gastrointestinal parasite species did not pose a serious health threat to seals in the Wadden Sea and seldom occurred in high levels of infection. No clear age related increase in infections was seen, contrary to findings by Strauss et al. (1991) and no strong correlation between parasites and gastrointestinal inflammation was found.

E. horridus prevalence (4%) on harbour seals was very low compared to the 39% found by Thompson et al. (1998) in the Moray Firth, off Scotland. However, in studies from the Netherlands (Borgsteede et al. 1991), Lower Saxony (Claussen et al. 1991, Strauss et al. 1991) and the Kattegat-Skagerrak (Lunneryd 1992), no ectoparasites have been reported at all. Although specimens can be washed off after the death of their host and might have been missed during necropsy the last record from the Wadden Sea was mentioned by Zimmermann & Nebel (1975), indicating that the prevalence of *E. horridus* has strongly decreased over recent decades. *E. horridus* probably carries low intensities of larval stages of *A. spirocauda*, which would account for the lack of *A. spirocauda* infection in seals infested with *E. horridus* (Conlogue & Ogden 1980). No lice and no *A. spirocauda* were found in 1999, which might underline the fact that they normally occur together.

The considerable variation in earlier data indicate a high spatial, individual or method based heterogeneity. Repeated parasitological surveys are needed to evaluate the fauna and prevalence over periods of time and in relation to the health status of harbour seals.

CONCLUSIONS

Metastrongyloid nematodes from the respiratory tract play a major role in causing disease and mortality in the Wadden Sea seal population, contrary to all other parasite species that were not found to be a major health threat to the seals. The parasite fauna and their prevalence differed significantly from data recorded during the first epizootic. While a much higher lungworm prevalence was found in this study there are decreasing trends to be observed in the prevalences of acanthocephalan and heartworm infections in comparison with studies from the 1988/89 epizootic as well as an increase in intestinal parasitation over the study period. Evaluating data from seals that died during the second PDV epidemic in 2002 in the Wadden Sea might show if the animal's immune system is suppressed by the virus and if it has any influence on the susceptibility of harbour seals to parasites.

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CHAPTER IV: TRANSMISSION OF LUNGWORMS IN PORPOISES AND SEALS: MOLECULAR TOOLS DETERMINE VERTEBRATE INTERMEDIATE HOSTS

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ABSTRACT

Porpoises and seals from German waters show infections by six species of lungworms belonging to the Metastrongyloidea. These nematodes parasitizing the respiratory tract are especially pathogenic and often cause secondary bacterial infections. In spite of the importance of lungworm disease for the health of wild populations and their clinical and epidemiological significance, the life cycle and biology of lungworms in the marine environment is still largely unknown. Regions of the ribosomal DNA (ITS-2) of all lungworms parasitizing porpoises and seals in German waters were sequenced to characterise and compare the different species. The phylogenetic relationship among the lungworm species was analysed by means of their ITS-2 sequences and the species-specific traits of the ITS-2 were used to screen possible intermediate hosts for larval stages of lungworms. Molecular markers to identify larval nematode stages via *in-situ* hybridisation in tissues of porpoise and seal prey were developed. Potential intermediate flatfish hosts from the North Sea were dissected and found to harbour nematode larval stages. Histological examination and *in-situ* hybridisation of tissue samples of these flatfish showed lungworm larvae within the intestinal wall. Based on larval ITS-2 nucleotide sequences the nematode stages were identified as *Pseudalius inflexus* and *Parafilaroides gymnuris*. Experimental infections of turbot (*Psetta maxima*) bred in aquaculture with live seal lungworm larvae (*Otostrongylus circumlitus*) were performed and second and third-stage larvae of *O. circumlitus* were isolated from their gastrointestinal tract. Our results show that vertebrate intermediate hosts play a role in the transmission of metastrongyloid nematodes of porpoises and seals.

INTRODUCTION

Parasite species infecting harbour porpoises (*Phocoena phocoena*) and harbour seals (*Phoca vitulina*) often cause secondary bacterial infections, inflammatory lesions and mortality (Jepson et al. 2000, Gosselin et al. 1998, Siebert et al. 2007). One particularly pathogenic group of parasites is represented by six nematode species belonging to the superfamily Metastrongyloidea that are associated with the respiratory tract (Lehnert et al. 2005, 2007). Metastrongyloid lungworms infect a wide range of mammals and are usually found in the bronchi, parenchyma or blood vessels associated with the lungs (Arnold & Gaskin 1974, Gosselin & Measures 1997). In marine mammals they often cause pneumonia, impede diving ability and are hence an important factor regarding the viability of wild populations (Measures et al. 1995, Gulland & Beckmen 1997, Siebert et al. 2001). Secondary bacterial infections due to parasites are a frequent cause of mortality in odontocetes (Gibson et al. 1998, Wünschmann et al. 2001) and pinnipeds (Dailey 1970, Onderka 1989). Lungworms are the most pathogenic metazoan parasites infecting marine mammals in German waters (Siebert et al. 2001, 2007) propagating secondary bacterial infections as frequent cause of death in porpoises (Lehnert et al. 2005) and especially in young seals (Lehnert et al. 2007). In harbour porpoises, four species of lungworms occur, all belonging to the Pseudaliidae (Metastrongyloidea), an ancient taxon restricted to toothed whales. These species (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*) differ in size and morphology and have adapted to different niches in the respiratory tract. Some species, such as *Stenurus minor* in harbour porpoises (*Phocoena phocoena*) and *Stenurus globicephalae* in pilot whales (*Globicephala melas*), also invade the tympanic bullae and cranial sinuses (Arnold & Gaskin 1974). In harbour seals, two species of lungworms occur (*Otostrongylus circumlitus*, *Parafilaroides gymnurus*) (Lehnert et al. 2007) that belong to two different families (Crenosomatidae and Filaroididae, respectively) members of which also parasitise terrestrial mammals such as carnivores and insectivores. The lungworms infecting marine mammals have evolved from ancestors infecting terrestrial hosts, and the survival of the ancient family Pseudaliidae in marine hosts is assumed to be due to the absence of competitors in the marine environment (Anderson 1982). A long period of co-evolution between these marine hosts (porpoises and seals) and their lung parasites can be assumed (Anderson 1982). Currently only little is known about the ecology and transmission of lungworms in the marine environment. Despite of the high general interest in marine

mammals and the known impact of lung parasites and associated diseases on the viability of their populations, descriptive studies on their parasite load predominate the available literature (e.g., Bergeron et al. 1997, Jepson et al. 2000, Gosselin et al. 1998, Gulland & Beckmen 1997, Onderka 1989). Lungworm transmission in marine mammals has been studied to a much lesser extent (Bergeron et al. 1997, Dailey 1970, Dailey et al. 1991, Houde et al. 2003), and data on the life cycle of lungworms infecting marine mammals in the North Sea are completely missing.

Most terrestrial metastrongyloids are heteroxenous, using invertebrates (molluscs, earthworms) as intermediate host (Anderson 2000). Experimental infections using metastrongyloids infecting marine mammals have indicated that they may use a vertebrate as intermediate host (Dailey 1970) while other studies found evidence for prenatal infections with lungworms in cetaceans (Dailey et al. 1991). The identification of parasitic strongyloid nematodes is usually based on morphological criteria. However, a number of biochemical and molecular studies have demonstrated the existence of genetically distinct but morphological similar (cryptic) species (Beveridge et al. 1993, 1994, Chilton et al. 1995), indicating that morphology alone may be insufficient for the unequivocal delineation of species. This is particularly the case for the eggs and larvae, where there are only a few or even no morphological characters to determine the species they represent. Several studies have shown for the strongyloid nematodes that the second internal transcribed spacer (ITS-2) of the nuclear rDNA genes provides genetic markers for species identification (Campbell et al. 1995, Hoste et al. 1995, Stevenson et al. 1995, Samson-Himmelstjerna et al. 2002). ITS-2 sequences can therefore be helpful for species identification in the absence of reliable morphological characters and be used for non-invasive diagnosis of parasite infections from faeces or blowhole swabs in live marine mammals, e.g. in rehabilitation. The aim of the present study was to characterise the second internal transcribed spacer (ITS-2) of the ribosomal DNA of seven lungworm species from harbour porpoises, harbour seals and pilot whales to be able to discriminate between species in all life history stages and to analyse the phylogenetic relationship among them. Subsequently, the ITS-2 sequences were used to gain information about the life cycle of these parasites. As potential intermediate hosts, several flatfish species from the North Sea were investigated for larval nematode stages because they are typical prey items of seals and porpoises. Larval nematodes were identified using the species-specific traits of their ITS-2. Flatfish bred in aquaculture were experimentally

infected with live first-stage lungworm larvae and moulted second and third-stage larvae were recovered from their gastrointestinal tract and identified by molecular tools.

MATERIALS AND METHODS

Collection of adult parasites

Lungworms were sampled during necropsies performed on stranded and bycaught porpoises and stranded seals that were examined in the frame of monitoring programmes to evaluate their health status (Siebert et al. 2007). Some animals found terminally ill were put to death by a shot to the head by authorised national park rangers or given a lethal injection by a veterinarian (Lehnert et al. 2007). The species differentiation was done based on morphological characteristics (Lehnert et al. 2005, 2007). Some animals were dissected after storage at -20°C for up to six months, few were dissected while fresh. Parasites were preserved in 70-90% ethanol or frozen at -20 or -70°C . DNA was isolated from adult specimens of *Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*, all infecting harbour porpoises, and from *Otostrongylus circumlitus* and *Parafilaroides gymnuris* infecting harbour seals. Individuals of *Stenurus globicephalae* (Pseudaliidae; Metastrongyloidea) came from a pilot whale (*Globicephalus melas*) stranded at the coasts of Schleswig-Holstein.

Collection of larval parasites

Fish were fresh bycatch obtained from a cutter trawling for shrimp in the German North Sea. The trawls came from three different locations within the North Sea: Sylt Outer Reef, west of the island of Helgoland and close to the rivermouth of the Eider. Out of the 188 dissected fish, 99 (53%) were plaice (*Platessa pleuronectes*), 55 (29%) were dab (*Limanda limanda*) and the rest consisted of gobies, rocklings, atlantic herring, whiting, dragonet, solenette, scaldfish, sandeel, flunder and lemon sole. The fish, measuring between 13 and 25 cm in length, were dissected and the gastrointestinal tract was opened. After removing its contents, the tissue was squeezed between two petri dishes and screened for parasites under a binocular (Olympus SZH10 Research Stereo). Measurements were taken using a digital camera (Olympus Camedia) attached to the binocular with Olympus DP-Soft Version 3.2 software on a personal computer. Live larvae were isolated from the intestinal wall or attached adipose tissue using pincers and spicules prior to DNA isolation.

DNA isolation, amplification, and sequencing

Genomic DNA from individual adult worms or individual larvae was isolated using a QIAamp Tissue Kit or QIAamp Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The lungworms came from porpoises and seals of different age classes, both sexes and from the North and Baltic Sea. The ribosomal ITS-2 was amplified from five to 15 individual adult worms per nematode species. For each individual worm, PCR and sequencing reactions were performed at least twice. Ribosomal ITS-2 was amplified by PCR using primers designed from the adjacent conserved 5.8S and 28S regions of *Caenorhabditis elegans* (GenBank accession number: X03680). Oligonucleotide primers were: 5'- GCA GAC GCT TAG AGT GGT GAA A-3' and 5'-ACT CGC CGT TAC TAA GGG AAT C-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 either using DYEnamic ET terminator sequencing chemistry (Applied Biosystems, Darmstadt, Germany), PCR products were directly sequenced using a MegaBACE 1000 capillary sequencer or were outsourced (Seqlab Göttingen). Some ITS-2 regions showed repeats in their structure and then PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) and a pCR4-TOPO vector to facilitate sequencing. Plasmid DNA was isolated using a NucleoSpin Kit (MachereyNagel, Düren, Germany) and then sequenced. Nucleotide sequences were edited and aligned using DNASTar (version 5.07/5.52) software. As a control 18S regions were amplified and sequenced from larvae encountered in the fish and negative controls without template were included in PCR reactions. Primer sequences delineated from *Caenorhabditis elegans* (accession number: X03680) were 5'-CCC GAT TGA TTC TGT CGG C-3' and 5'-GGC TGC TGG CAC CAG ACT TGC-3'.

Phylogenetic analysis

Data collection

One dataset was compiled using the ITS-2 sequences obtained from the eight lungworm species infecting porpoises and seals in German waters and *Stenurus globicephalae* (Pseudaliidae) from a pilot whale. Sequence data of other nematode families and outgroup

taxa were downloaded from GenBank (see Table 1 for lists of species included in the dataset and GenBank accession numbers).

Alignment

The newly identified parasite ITS-2 sequences were aligned with other nematode ribosomal sequences using MAFFT version 6 (Kato et al. 2002). The program GBlocks v. 0.91b (Castresana 2000) was used to exclude unreliably aligned positions.

Phylogenetic analysis

Stationarity of nucleotide frequencies was estimated for all nucleotide datasets using a χ^2 test under the base frequencies option in PAUP*4b10 (Swofford 2002). Two species from nematode families Heligmosomatidae and Syngamidae served to root the trees of all analyses. Treepuzzle (Strimmer & von Haeseler 1997) was used for a Likelihood mapping analysis of the sequences.

Nucleotide substitution models were selected for the dataset using the Akaike Information Criterion (AIC) implemented in Modeltest 3.07 (Posada & Crandall 1998). Bayesian analyses were conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Nucleotide models were set according to the results obtained with Modeltest.

Maximum Likelihood (ML) analysis was conducted using RAxML (Stamatakis 2006) with the selected GTR + Γ + I model of sequence evolution. Clade stability was estimated by 100 replicates of non-parametric bootstrapping.

Two independent runs, each with four Markov chains, were run in parallel (starting each from a random tree) for 100,000 generations with trees being sampled every 500 generations. An average standard deviation of split frequencies < 5% was used as the indication for convergence. After convergence of likelihood, trees were discarded as *burn in*, and posterior probabilities were estimated as the frequency of clades in the trees sampled after standard deviation of split frequencies fell below 5%.

Experimental infections of fish

The fish species (turbot, *Psetta maxima*) used in the experiment was chosen because of its availability, ease of maintenance, and role in the diet of porpoises and seals (Benke et al. 1998, Sievers 1989). Aquaculture-bred turbot (*Psetta maxima*) (22-25 cm, age 1 year, n=18) were orally infected with live lungworm larvae and parts of the larvae and egg-filled uteri that were dissected from adult gravid female specimens of *O. circumlitus*. The nematodes came from three free-ranging yearling seals from the North Sea population that had to be

shot because of serious illness. Larvae were active when placed in petri dishes containing seawater where they were kept at 4°C prior to experimental infection. Six individual turbot were kept and infected at one time in a 70 l aquarium with ozonised sea water. Fish were intubated with live ~50 first-stage *O. circumlitus* larvae using a flexible plastics disposable pipette introduced into the oesophagus. Fish were killed at various time intervals (2 days post infection to 14 days post infection) using cervical dislocation (animal experiment permit) and dissected. The gastrointestinal tract of the fish was pressed between two petri dishes and examined with a binocular (Olympus SZH10 Research Stereo). For histological examination and *in-situ* hybridisation sections of infected intestine from several turbot were treated as mentioned above.

Histology and *in-situ* hybridisation

Intestinal tissue samples of different fish species were fixed in 10% buffered formalin, embedded in paraffin wax and proceeded for further investigations. For histology tissue slides were stained with hematoxylin and eosin (HE). *In-situ* hybridisation using the obtained RNA-probes was performed according to Gröters et al. (2005).

Tissue sections of fish intestine infected with larvae were subjected to *in-situ* hybridisation. The same sections of infected fish intestine had also been subjected to DNA isolation of larvae. Species-specific probes were designed using the ITS-2 nucleotide sequences of each lungworm species. Initially, a PCR was performed applying species specific primers. For *Pseudalius inflexus* (Metastrongylidae) the primers used were 5'-ATA GAA ATC ATC GTT GTC-3' and 5'-GTT CAG CGG GTA ATC AC-3' and for *Parafilaroides gymnurus* (Filaroididae): 5'-GTG GAT GCA TGG AGA ATG AA-3' and 5'-GTG CGA GTA CCG TTT GAC C-3' amplifying a PCR product of 204bp and 171bp length, respectively. Subsequently, PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen GmbH, Karlsruhe, Germany). Thereafter, templates were generated by PCR using reverse specific primers in combination with primers complementary to the M13 forward priming sites of the vector. These templates contained the T7-RNA polymerase binding site and the specific fragment of the species specific ITS-2 gene. After purification, Dig-labeled RNA probes were transcribed *in vitro* using T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany). The detection system consisted of an anti-DIG-antibody conjugated with alkaline phosphatase (1:200; Roche Diagnostics) and the substrates nitroblue tetrazoliumchloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, X-Phosphate; both Sigma-Aldrich, Taufkirchen, Germany).

Formalin-fixed and paraffin-embedded adult specimens of *P. inflexus*, *T. convolutus*, *H. invaginatus*, *S. minor*, *O. circumlitus* and *P. gymnurus* served as controls.

RESULTS

The ITS-2 sequences of the seven lungworm species were submitted to GenBank (accession numbers see Table 1) and aligned for phylogenetic analysis (see alignment in Appendix 1). *Stenurus minor* ITS-2 was cloned because of repetitive parts in the sequence. Sizes of the ITS-2 varied from 526 bp (*P. inflexus*) to 649 bp (*P. gymnurus*). No differences in the sequences were detected between worms of the same species originating from different geographic localities.

Table 1: Nematode species GenBank accession numbers and sequence sizes

Species	GenBank accession number	Sequence size basepairs
<i>Hovorkonema variegatum</i>	DQ679968	313
<i>Heligmosomum mixtum</i>	DQ408626	987
<i>Mecistocirrus digitatus</i>	AB114420	258
<i>Ostertagia ostertagi</i>	AB245021	1128
<i>Aelurostrongylus abstrusus</i>	DQ372965	523
<i>Metastrongylus pudendotectus</i>	AJ305383	466
<i>Metastrongylus elongatus</i>	AJ305381	501
<i>Metastrongylus salmi</i>	AJ305404	518
<i>Varelostrongylus alpenae</i>	AY648407	556
<i>Umingmakstrongylus pallikuukensis</i>	AY648409	472
<i>Pseudalius inflexus</i>	FJ767935	526
<i>Torynurus convolutus</i>	AY464532	612
<i>Halocercus invaginatus</i>	FJ787301	621
<i>Stenurus minor</i>	FJ787302	624

<i>Stenurus globicephalae</i>	FJ787303	577
<i>Otostrongylus circumlitus</i>	AY491979	570
<i>Parafilaroides gymnurus</i>	FJ787304	649

Intra- and interspecific variation

To examine intraspecific variation, five to fifteen separate DNA preparations per species from different individual nematode specimens were performed and sequenced. The ITS-2 sequences of the seven nematode species showed no intraspecific variation.

Interspecific variation between ITS-2 sequences investigated in this study showed highest similarities between *Torynurus convolutus* and *Stenurus minor* (87.9% identity) and the lowest between *Parafilaroides gymnurus* and *Pseudalius inflexus* and *Stenurus globicephalae* (46.8% identity), respectively (calculated with MegAlign, Clustal W, DNASTAR, see Table 2).

Table 2: Percent identity between the seven lungworm species investigated, calculated using Clustal W in MegAlign (DNASTAR version 5.07/5.52)

Percent Identity	<i>O. circumlitus</i>	<i>P. gymnurus</i>	<i>P. inflexus</i>	<i>T. convolutus</i>	<i>H. invaginatus</i>	<i>S. minor</i>
<i>P. gymnurus</i>	62.1	X	X	X	X	X
<i>P. inflexus</i>	52.1	46.8	X	X	X	X
<i>T. convolutus</i>	54.2	53.2	63.7	X	X	X
<i>H. invaginatus</i>	49.1	49.3	63.3	61.8	X	X
<i>S. minor</i>	54.9	53.0	65.6	87.9	66.0	X
<i>S. globicephalae</i>	51.6	46.8	71.5	70.1	55.2	68.9

Phylogenetic analysis

Bayesian inference and ML-analysis recovered congruent trees (Fig. 1). Analysis of our ITS-2 dataset indicated insignificant ($p = 0.8796$) stationarity of nucleotide frequencies among lineages. In all analyses, we recovered paraphyletic Pseudaliidae, with *P. gymnurus* (Filaroididae) clustering in this group as sister taxon of *H. invaginatus* (ML-Bootstrap 87%). Protostrongylidae appeared also paraphyletic with respect to Crenosomatidae, but we got no clade support for this grouping.

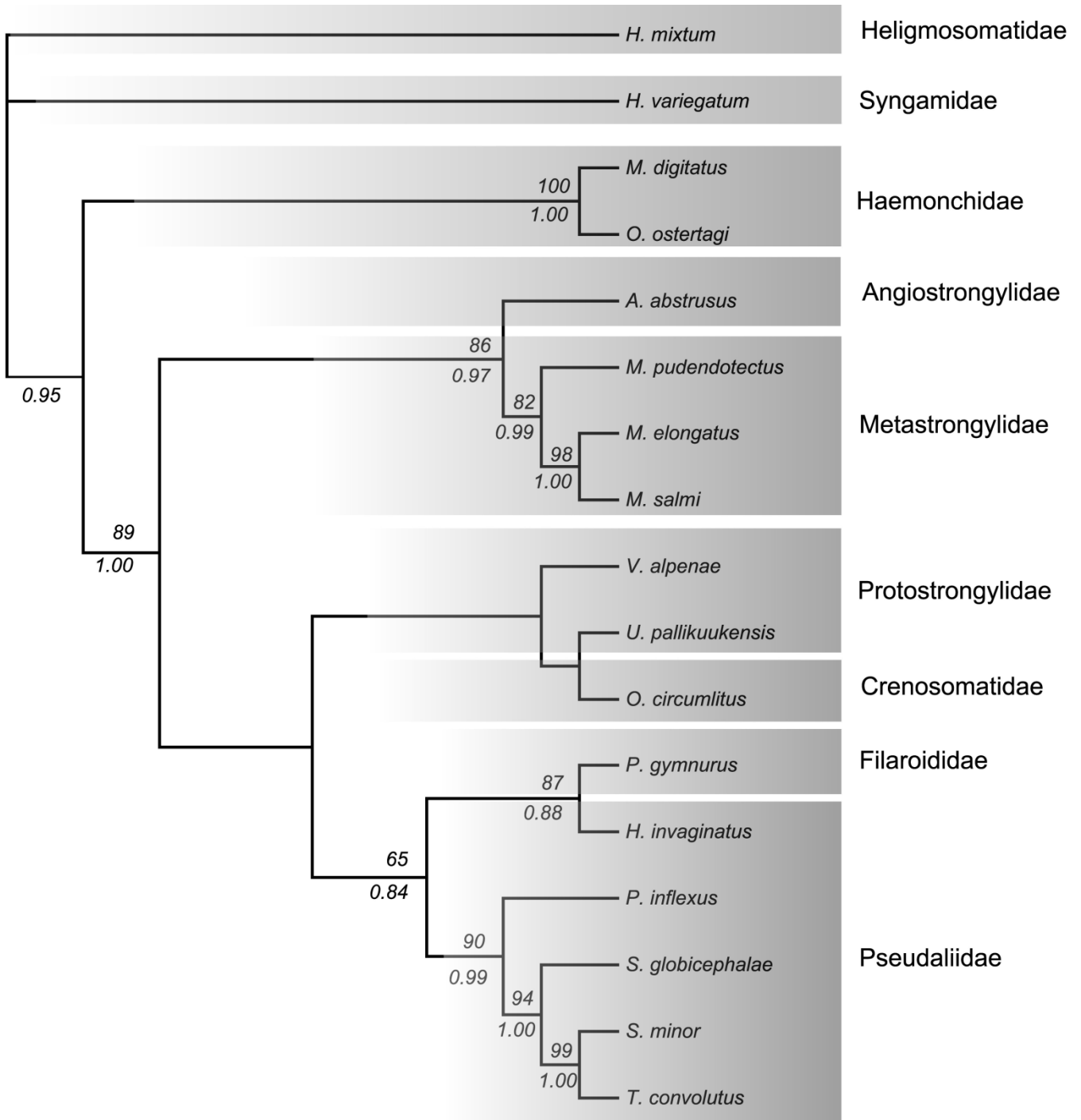


Figure 1: Maximum Likelihood analysis of the ITS-2 dataset using raxML with GTR and cat (gamma approximation) + I. Bootstrap support estimated from 100 replicates is given above the branches. Posterior probabilities estimated with MrBayes using the same model are given below the branches. Heligmosomatidae and Syngamidae root the tree.

Fish dissections

From those 30% (57) of fish that had larval parasite stages in their intestinal wall, 58% (33) were dab and 37% (21) plaice. One witch (*Glyptocephalus cynoglossus*) and two lemon soles (*Microstomus kitt*) were also infected with larval nematode stages in small numbers in the intestinal wall. Larvae in infected fish occurred throughout the whole length of the intestine and in numbers from one to hundreds. Few were found in the mesenteric adipose tissue attached to the intestine. They measured less than one mm in length (between 0.3 and 0.7 mm), and between 0.01 to 0.02 mm in width, and stayed alive for at least two days when kept at 4°C. Most larvae were moving in a sheath, indicating that they had passed through a moult in the intestinal wall (second-stage larvae). Third-stage larvae were found located in their first and second-stage larval cuticles after their two moults (Fig. 2). Species identification based on morphological characteristics was impossible due to the tiny size of the larvae.

DNA was isolated from 133 individual larvae and followed by amplification of ITS-2 with our specific primers. From 61 (46%) larvae, PCR products could be generated and produced a band in electrophoresis corresponding to the expected ITS-2 (~500-700bp) fragment length. After sequencing, 41 (31%) larval nematode stages could be identified according to their ITS-2 sequences. The 41 larvae originated from 25 individual fish that came from all three sampling locations. Twenty *Parafilaroides gymnurus* larvae were isolated from 9 individual plaice and one dab. Sixteen *Pseudalius inflexus* larvae were isolated from seven individual dab and six different plaice. Four *Torynurus convolutus* larvae were identified from three individual dab. One *Stenurus minor* larva was determined from a dab that also harboured *Pseudalius inflexus* larvae. In one dab *Pseudalius inflexus* and *Parafilaroides gymnurus* larvae occurred together. For the first time fish intermediate hosts for these lungworm species were identified from the wild. 20 DNA preparations from larvae in fish intestines produced a band in electrophoresis corresponding to the expected ITS-2 fragment length but the PCR products did not perform in the sequencing reactions or sequences were of poor quality.

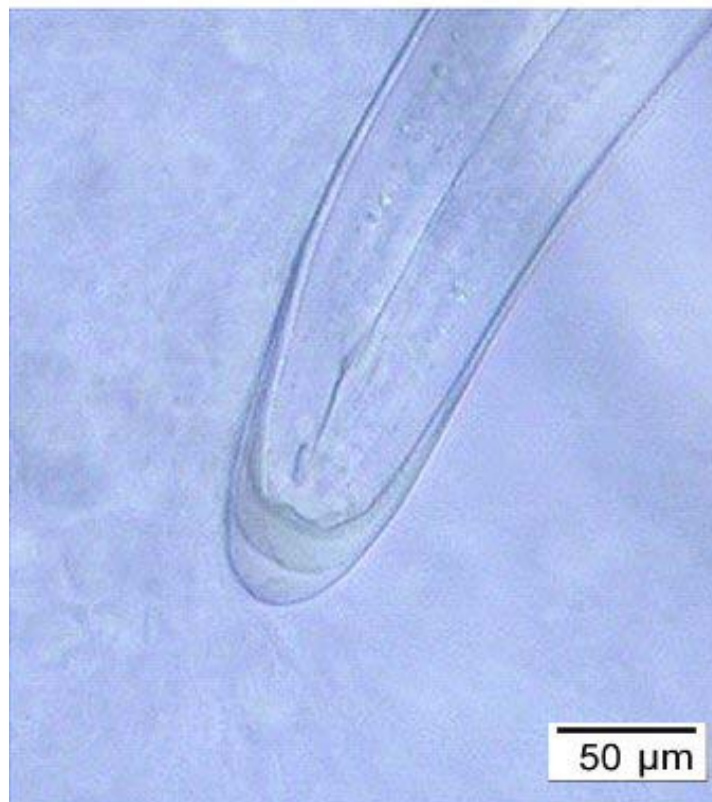


Figure 2: Third-stage lungworm larva located in first and second-stage larval cuticle situated in intestinal mucosa of plaice (*Pleuronectes platessa*).

Experimental Infections

In seven (40%) of 18 turbot experimentally infected with larvae dissected from the seal lungworm *O. circumlita*, larvae were found in the intestinal wall. DNA was isolated from 19 larvae originating from the seven experimentally infected turbot. PCR products were sequenced and aligned to ITS-2 sequences derived from adult lungworm specimens. They were identified as *O. circumlita*. This is the first study to show that turbot is a suitable intermediate host for *Ostertongylus circumlita*.

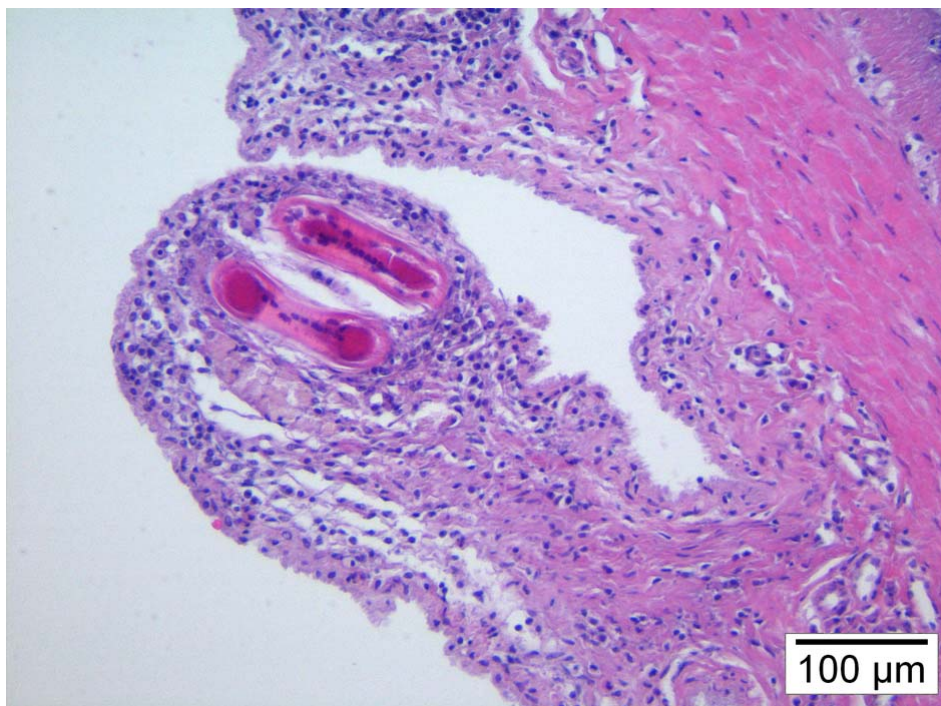


Figure 3: Histological slide of dab (*Limanda limanda*) intestine with lungworm larval section located in mucosa. 20fold magnification.

Histology

The histological examination of the intestinal tract in several naturally and experimentally infected fish revealed larvae within the lamina propria of the intestinal mucosa (Fig. 3, Fig. 4A). In addition, larvae were also detected in adjacent tissues including mesenterium and pancreas (Fig. 4B). Due to poor tissue preservation in some of the intestines of the caught fish, an exact determination of the topography of the larvae could not be performed in all intestines. Larvae were surrounded by a mild to moderate chronic granulomatous inflammation consisting of macrophages, lymphocytes, few multinucleated giant cells and fibrosis (Fig. 4A). However, several larvae were found without any inflammatory reaction in the surrounding tissue (Fig. 4B).

***In-situ* hybridisation**

After applying an RNA-probe directed against *Pseudalius inflexus* (PiT7) to the positive controls (adult worms of all six lungworm species), a positive reaction with all specimens was noted indicating that this probe is not suitable for the discrimination between different lungworm species. In contrast, the RNA-probe for *Parafilaroides gymnurus* (Pgp639) showed a positive reaction only with *Parafilaroides gymnurus* and was therefore considered suitable for species identification. In all worms, predominantly cells of the intestinal tract were labelled.

Within the intestinal tract of the caught fish labelled larvae were found in the intestinal wall in 9 out of 10 samples using the PiT7 probe. However, using the Pgp639 probe only larvae in the intestinal wall in 3 out of the 10 samples showed a positive reaction.

Within the experimentally infected fish labelled larvae were found in 2 of 6 samples. PiT7 detected larvae in the lamina propria of the intestinal tract (Fig. 4C) and also in the adjacent mesenterium and pancreas (Fig. 4D), whereas with Pgp639 only larvae within the mesenterium and pancreas were positive (Fig. 4E; 4F).

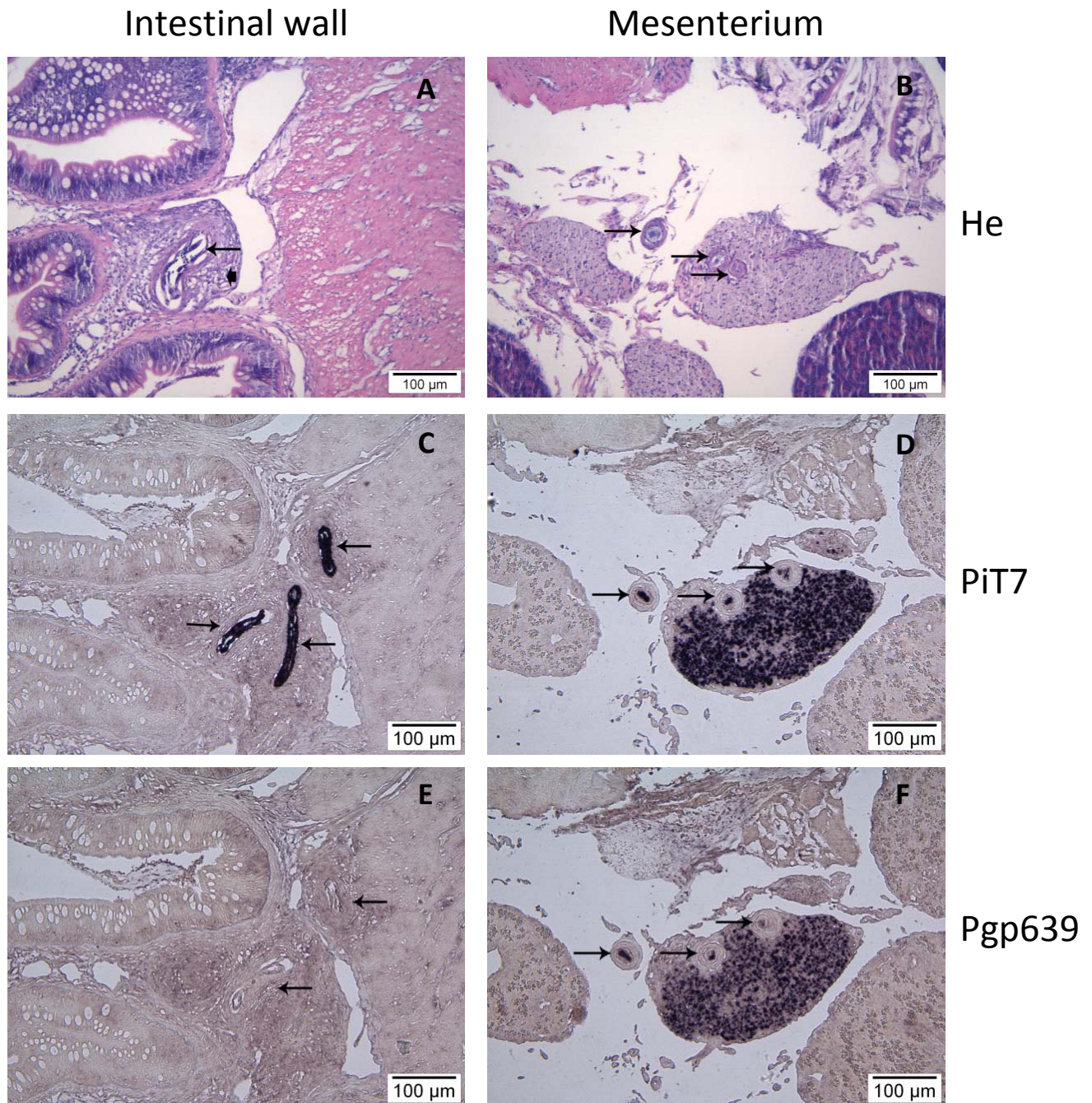


Figure 4: Experimentally infected fish A: Intestinal wall, larvae in the lamina propria of the intestinal mucosa (arrow) surrounded by a moderate chronic granulomatous inflammatory reaction (arrowhead), hematoxylin and eosin staining. B: Mesenterium, larvae in the mesenterium adjacent to the intestine without inflammatory reaction (arrows), hematoxylin and eosin staining. C: Intestinal wall, labelled larvae in the lamina propria of the intestinal mucosa (arrows), *in-situ* hybridisation using PiT7 probe. D: Mesenterium, labelled larvae in the mesenterium adjacent to the intestine (arrows), *in-situ* hybridisation using PiT7 probe. E: Intestinal wall, unlabelled larvae in the lamina propria of the intestinal mucosa (arrows), *in-situ* hybridisation using Pgp639 probe. F: Mesenterium, labelled larvae in the mesenterium adjacent to the intestine (arrows), *in-situ* hybridisation using Pgp639 probe.

DISCUSSION

Intra- and interspecific variation

The marine lungworm ITS-2 sequences under investigation in this study showed no intraspecific variation. This is concordant with several studies reporting no intraspecific variations in strongylid nematodes like *Haemonchus* spp. and *Trichostrongylus colubriformis* (Campbell et al. 1995, Hoste et al. 1995, Stevenson et al. 1995) and confirms the general low or missing incidence of intraspecific variation in the ITS region. Interspecific differences within the ITS-2 of the investigated seven lungworm species are clear and sufficient for an unequivocal distinction between the species.

In view of the observed low variability in the ITS-2 within species and the clearly represented spectrum of lungworm species in porpoises and seals a species-specific PCR and RFLP assay might be established for rapid species identification of larvae and eggs of lungworms in the future.

Fish dissections

There are many reports of unidentified larvae in fish (Margolis & Arthur 1979, McDonald & Margolis 1995) and e.g. larvae in the intestinal wall of flatfish have been reported by Kjøie (2000, 2001). They were detected by using a dissecting microscope and some larvae in the gut wall of benthic fish were identified morphologically as belonging to the nematode family Cucullanidae (Kjøie 2000, 2001) which spend one stage of their life history in the intestinal wall of their fish final host. Others recorded 'unidentified larvae' (El-Darsh & Whitfield 1999) from flatfish off the British coast that might be the larval metastrongyloids identified in this study. In the present study sometimes PCR reactions using the specific ITS2-primers with DNA from larvae in fish intestines yielded no products and no bands in electrophoresis (n=72). A reason may be that in these cases the amount of DNA isolated was not sufficient for PCR reactions or that the DNA originated from larval stages of other parasite species, e.g. *Cucullanus spec.* (Kjøie 2000, 2001).

Most larvae isolated from plaice were *Parafilaroides gymnurus* while larvae from dab consisted of several lungworm species. In general, dab were more frequently infected by larval nematode stages. It can be presumed that dab is an intermediate host for some of the lungworm species under study here, while plaice is more specifically used by *P. gymnurus*. Other fish species investigated do not appear to be suitable intermediate hosts for

metastrongyloids. Larvae found in one witch (*Glyptocephalus cynoglossus*) and two lemon soles (*Microstomus kitt*) could not be identified.

It remains to be investigated if additional invertebrate paratenic or transport hosts (e.g. ophiuroids or polychaets) are involved in the transmission of first-stage larvae to flatfish.

Experimental infections

40 percent of the turbot were successfully infected in the experiment. Some of the turbot regurgitated after being infected orally and food pellets they were fed, larvae and nematode tissues floated in their holding tanks. The live lungworm larvae might not have been ingested and infection might have failed in those cases. However, a third flatfish intermediate host was identified. The experimental infection showed that *O. circumlitus* represents the third lungworm species in this study that uses flatfish as intermediate hosts in their life cycle. No transport or paratenic host and/or invertebrate were involved in the transmission from first-stage larvae to the flatfish under experimental conditions. In the three existing studies trying experimental infections with metastrongyloids from marine mammals, American plaice (*Hippoglossoides platessoides*) has been reported to act as intermediate host for *O. circumlitus* from ringed seals (Bergeron et al. 1997) and American plaice and Arctic sculpins (*Myoxocephalus scorpioides*) as intermediate hosts for *Pharurus pallasii* from beluga whales in Canada (Houde et al. 2003). *Girella nigricans*, a coprophagic fish, has been found to play a role in the transmission of *Parafilaroides decorus* in California sea lions (Dailey 1970). In none of these studies molecular tools were used and the identification of larval nematode stages was attempted using morphological characteristics or by criterion of exclusion.

Histology

Histology revealed larval stages of the lungworms in different locations including mucosa, mesentery and pancreas. The inflammatory reaction was generally mild to moderate or lacking, which indicates some degree of immunological tolerance. The presence of larvae outside the intestine points to active somatic migration. Identification of larvae of different lungworm species using morphological criteria or their tissue location is not possible. Larval nematode stages, identified morphologically as *P. decorus*, situated in different parts of the intestinal wall and in the mesenteric adipose tissue attached to the intestine were found by Dailey (1970) in a coprophagous fish (*Girella nigricans*) in California. Bergeron et al. (1997) and Houde et al. (2003) recorded larvae of *P. pallasii* and *O. circumlitus* in the intestinal wall in American plaice and Arctic sculpin after experimental infections. Our results are

concordant with previous studies and indicate that larval stages of lungworms migrate through the intestinal wall of their intermediate hosts during their development.

***In-situ* hybridisation**

In-situ hybridisation applying the PiT7 probe revealed in all specimen where larvae were found a positive result indicating that the PiT7 probe shows a cross reactivity with all investigated worm species. In contrast, Pgp639 only labelled *Parafilaroides gymnurus* larvae in 3 of 10 fish without noticeable cross reactivity to the other worm species apart from *O. circumlitus*. However, it was not possible to find labelled larvae in all tissue sections. This is presumably due to an absence of worms from the slides used for *in-situ* hybridisation since unlabelled worms were also not detected on these slides. Our results show that we have successfully developed a tool to classify metastrongyloid nematodes from histological slides. The probes not only enable us to screen tissues of potential intermediate hosts for larval nematode stages but can be used to trace the route larvae take migrating from the intestinal tract of the final marine mammal hosts presumably via the lymphatic system to the respiratory tract where they become mature. Histological sections of lymph nodes of porpoises and seals showing nematode infections will be investigated using these markers. These analyses will further elucidate the life cycle of metastrongyloids. Additionally, molecular markers will be used as diagnostic tools in the future to determine lungworm infection in various organs of intermediate and final hosts, e.g. in routine histological investigation of lung tissue slides.

CONCLUSIONS

In this study ITS-2 sequences of lungworms from porpoises and seals from German waters were characterised and the data used to describe the phylogenetic relationship among this group of metastrongyloid nematodes. Molecular data were used to study the life cycle of these parasites and identified flatfish as vertebrate intermediate hosts. For the first time the heteroxenous life cycle of the rarely studied lungworms of porpoises and seals was documented and an important contribution to the knowledge of their ecology achieved. The newly developed molecular tools to identify larval lungworm stages may be refined and used as diagnostic tool for the diagnosis of parasites in live marine mammals from faecal samples and blowhole swabs. They will help to define the life cycle of the lungworm species in more detail when tissue samples from dead stranded animals are screened and the migration path

of larvae from the intestinal tract of the final host to its respiratory tract is tracked. Molecular data are highly suitable for the identification of larval nematode stages when morphological characterisation is no option.

This is the first study to determine intermediate hosts for the investigated metastrongyloid nematodes *P. inflexus* and *P. gymnurus* of marine mammals in the wild. It documents for the first time the transmission and heteroxenous life cycle of two lungworm species of seals and porpoises in the Wadden Sea.

With our successful experimental infection of turbot we have found another flatfish intermediate host for a third lungworm species.

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Appendix 1: Alignment using CLUSTAL (-like) formatted alignment by MAFFT (v6.500a)

```

Oc          tgaacgcata-gtgcgctgggatttcccttcggcacatctggttcagggttgt-taatt
Pg          tg-acgcata-gcgtcgttgggttttcccttcggcacatctggttcagggttgt-taaaa
Pi          tgaacgcata-gcgtcgttgggttttcccttcggcacatctggttcagggttgt-taaaa
Sm          tgaacgcata-gcgtcgttgggttttcccttcggcacatctggttcagggttgt-taaat
Tc          tgaacgcataaggcgtcgttgggttttcccttcggcacatctggttcagggttgt-taaat
Sg          tgaacgcata-gcgtcgttgggttttcccttcggcacatctggttcagggttgtgaaaaat
Hi          tgaacgcata-gcgtcgttgggttttcccttcggcacatctggttcagggttgt-taaaa
           ** ***** * ***** *****

Oc          attataactacagtcgtata-atatgcgta-----
Pg          atcataaatatagtctcgtgtgtatgtgtattgtatacgtggatgcatggagaatgaacg
Pi          atcaataatacaatcgtatatatg-----
Sm          atcatgaatacattcgtctatgtgtatgta-----
Tc          atcatgaatacgttcgtctatgtgtgtg-----cgygt
Sg          atcatgaatacattcgtctatttgtga-----
Hi          atcaacaatatagtcgtat-tgtacacgtg-----gaggt
           ** * * * * ** * *

Oc          -----tgattgtgtattatcgtatattatttgaataaaat
Pg          tgatgcataccattaatggtggctgtgaataggaaaagaagtgactaatcgtaggcactt
Pi          -----
Sm          -tgtgtgtgtagtgtgtggcgtttgtgaacacac-----
Tc          gtgtgtgtgtagtgtgtggtgtgtgtgaacacatatatacaca-----
Sg          -----
Hi          agacgaacg-----gcaaatgaacacataagcaagtatgtgtcgttcatcata-

Oc          gcatgacatagcgttttaaaaaattctctatatgcgatatacatatacatgtata-----
Pg          ttcttagcatgacggttcacgttttctcgttatatgaatgcgtttcacatataca-----
Pi          -----
Sm          -----acacacatatatatatatatgcat
Tc          -----cacacaacacacacacacacacacacatctgcat

```

Sg -----cgg-----
 Hi -----ggtcgtcgcgttctacgtgtgcggc-----cagatatgat-----

Oc gttatgtgactgtttgtcaaacgggtactcag-tcata-----ccagcattaa
 Pg ---cgatgactgttggtcaaacgggtactcgc-actta-----
 Pi ---tgacgattgtttgtcaaacgggtactcgt-acgta-----
 Sm gtgtggcgaatgttcgtcaaacgggtactcga-tcgtg----tacgtgcacacttgatt--
 Tc gtgtggcgaatgttcgtcaaacgggtacttgc-tcgtg----tacgtgcacacttgatt--
 Sg ---cggcgaatgttcgtcaaacgggtactcgc-tcgtagcgggtgtgcacacattgtgtgt
 Hi ---acgcgactgttggtcaaacgggtactcgcgtcata-----

* * * * * * * * * * * * * * * * * *

Oc tattgtcgggtaatttgatacccggtttta--gtaa--agaatgaacagattacaaaatat
 Pg -tcatcgatatgag-cgattcccggtttta--gtga--tgatttaaaggatagcaacatgt
 Pi -tcgtcgatatgag-cgattcccggttttagttttaatgatttaaaaagatagcaacatgt
 Sm -gttgtgatacgagccgattcccggtttta--gtgaacagattgaaacgatagcaacatgt
 Tc -gttgtgatacgagccgattcccggtttta--gtgaacagattgaaacgatagcaacatgt
 Sg gtgtgtgatacgaggcgattcccggttttagtgtgaacagattgaaacgatagcaacatgt
 Hi -----tcgatgcgattcacggtttta--gtaa--tgattgaaatgatagcaacatgt

* *

Oc acaga-acacgtatctgtatatgt-----gttttatgtcgaatgtgatataaa
 Pg aatatgata-----ttattgaactgaatgtgatcgtat
 Pi aatattatatatgtatatatatatatatcacgtatgatattacgccgaatataatgtgtat
 Sm tataat-atatatatatatatatatatatat---atattacaccgaatataatgtgtat
 Tc aatat-aca-----atagataatgtgt---agattacaccgagtataatgtgtat
 Sg aatat-ata-----cgattttatatatgtat---atattacaccgaatataatgtgtat
 Hi aatag-tag-----aatttctat-----tattacaccgaatgtaatgtgggt

* * * * * * * * * * * * * * * * * *

Oc cgttgatgtgtatgtgtagatatatgtatgcatagatatgctatgttatatagtgaagat
 Pg aa---atgtgtg-----aatatgt-----tagttatgtcat-----tac
 Pi gatgaatgtgtg--tgtatatatgtat-----tagttgtgtcat-----tat

```

Sm      ga---gtgtgtg--tgtgaatacgtgt-----tagttatgtcat-----cat
Tc      ga----gtgtg--tgtgaatatgtgt-----tagtcatgtcat-----cat
Sg      ga---gtttgtgaatatgtgtgtgtgt-----tagttatgtcat-----tac
Hi      ga---atgtgtg--tatgtgtgtgtgt-----gtgtta-atcag-----tgt
          ***          * *          *          *

Oc      tattg-ctatcattgtcgaaa-----tgattttca-----aat
Pg      tttcg-tcgtcatctttgacgta-----gtgattttca----atggg-----
Pi      tadc-----gcgttgttgatg-----ttgattttca----atggg-----
Sm      ta----ctaccgttgttggcgtggcgtgtattgattttt-tcatcgtgga-----
Tc      tact--ctaccgttgttggcgttagcatgtattgattttcatcgtggtgga-----
Sg      tacctaccatcgttgttggcgttagcgtg--ttgattttcatcgtcgtacagagacatcat
Hi      catt--acacagtgatcagatgatgacg--atgattttca----atggg-----
          *  *          * * * * *

Oc      gtcgttatgcttgaaaatcata-----tat
Pg      ----taccattgaaagtcgtgaaatgtatttt-----t
Pi      ----taccgttgaaaatcatagaaatcatcat----cgttgcgtttttcatgtgacat
Sm      ----taccgttgaaaatcgaagacgca-----tgcgtgatgc
Tc      ----taccgttgaaaatcgtatagacgcacatt-----ttcatgtgcgtgatac
Sg      ctcatcgtcgttgaaaatcgaagacgtacatcacattttcacatgagtgtgcgtgccat
Hi      ----taccgttgaaagtcgtgaaat-----tgttgt
          *  * * * * *  **

Oc      attcgac-----aataatattaca
Pg      cattgccgttggatggtgatgaaatgcataattattg-----agtagtgacaata
Pi      catcgttatcgttatcggtaatggatgtgatgtttgttgaaa-----tgtaatgacaata
Sm      catcgat-----ggattgattg-----agtgatgacaata
Tc      catcgat-----ggattgattg-----agtgatgacaata
Sg      catcgat-----ggatgtgcgttaattg-----agtgatgacaata
Hi      cgtcgtt-----gatcagcttttagctgtgaattttggcatcgttgata
          *  *          *  *

Oc      t-----attacacatggt-----tagaacgtatgtataattcacatat

```

Pg a-----atactatacatatg-----gcgatggcacacaaggatattttcttcaa
 Pi a-----atactatacatatagcgagcaatatagcacacaaattta----aataa
 Sm t---gcatatacaactacatgtatg-----tacatatcacacaaacacattaataata-
 Tc t---gcatatacgactacatgtata-----cacat----cacaaacacattaataatg-
 Sg tgtggcgtatacgactatatacata-----cacata---tatgaacacacaaaccacat
 Hi t---tgatatgtagcaccacatctaatatatcgaatttcattcaccaccacatttcatta

* * * *

Oc catttttttttt-----ttgttgtatatgcaacctgagctcagatgtgattacc
 Pg tacgtatctcattagaaat----tctatatatacaacctgaaactcagacgtgattacc
 Pi tacacattttcattggaatattttctataaatgcaacctgaaactcaaattgtgattacc
 Sm -----tttgaaatgcaacctgaaactcagatgtgattacc
 Tc -----tttgaaatgcaacctgaaactcagatgtgattacc
 Sg tagaatgcattta-----ttatacaagaatgcaacctgaaactcagatgtgattacc
 Hi ttgtt-----aatgcaacctgaaactcaggtgtgattacc

** ***** ** *

Oc gctgaact
 Pg gctgaact
 Pi gctgaact
 Sm gctgaact
 Tc gctgaact
 Sg gctgaact
 Hi gctgaact

GENERAL CONCLUSION

This thesis aimed at characterising parasite fauna and its impact on the health status of harbour porpoises and harbour seals in German waters. Additionally, it intended to gain insights into the life history of their lungworms by combining methods from traditional parasitology with DNA technology and newly developed molecular tools.

Information is presented on parasite species and associated diseases in harbour porpoises (chapter I & II), parasitism in seals related to the PDV epidemics (chapter II) and the ecology and life cycle of the lungworm species infecting porpoises and seals (chapter IV). New data about parasite species infecting porpoises and seals were collected and the understanding of their importance for the health of these animals was improved. Information about the so far unknown life cycle of the lungworm species was gained.

Each of the four chapters provides important new data on parasitism in porpoises and seals and improves our knowledge about the biology of host and parasite.

In the following, a synthesis is given that addresses the following questions, to the extent that they are not discussed within the dedicated chapters:

- 1) What are the overall patterns of parasitic infections in porpoises and seals?
- 2) What are the consequences of parasitism for porpoises and seals?
- 3) What can be gathered from the new data about lungworm ecology and how can they be used for future research?

(1) What are the overall patterns of parasitic infections in porpoises and seals?

Numerous parasitological studies have been carried out on marine mammals (see Geraci et al. 1987, Raga et al. 1997) but few were dedicated to parasites of porpoises and seals from the German North and Baltic Seas (Brosens et al. 1996, Lick 1991). This thesis gathered baseline data on the parasite spectrum of harbour porpoises and harbour seals and examined the impact of the different species on the health status of their hosts.

Most porpoises and seals investigated in this study were parasitised by several parasite species and in different organ systems at the same time. Most species were endoparasites and the majority belonged to the order Nematoda. Harbour porpoises as well as harbour

seals commonly showed parasitic infections in the respiratory and alimentary tract with different nematode species cohabiting the same organs. The parasite fauna and their prevalences differed significantly between the two host species, indicating strong host specificity of the parasites. None of the parasite species occurred in both marine mammal species (chapter I, II & III), although the hosts are sharing habitat and are known to prey on the same fish species (Bjørge & Tolley 2002, Gilles et al. 2008). For the first time, two species of lungworms (*Halocercus invaginatus*, Pseudaliidae and *Parafilaroides gymnuris*, Filaroididae) not previously recorded from German waters were found in this study. Both are very small and fragile species which, due to their location and size, are difficult to diagnose and remove from the lung tissue of their host. Harbour porpoises were infected by four different lungworm species and seals by another two; only one stomach nematode infected porpoises while seals were infected by two to three different species. Harbour porpoises, in contrast to seals, were infected by nematodes in the tympanic bullae and by trematodes in the last stomach compartment, liver and pancreas. Although trematodes in the liver have been reported for seals (Strauss et al. 1991), none have been found to infect seals from German waters during this study. The occurrence of a heartworm in seals, which is not mirrored by anything similar in porpoises, may have its reason in the still amphibian lifestyle of pinnipeds. Seal lice, which are also dependent on land to complete their life cycle, are believed to be the vector for their transmission (Geraci et al. 1981). Ectoparasites were found to occur on both host species, however, they belonged to two different orders, the Insecta and the Crustacea, and their respective life histories are completely different. While the seal louse was derived from terrestrial ancestors of the pinnipeds and has adapted partly to the marine environment, the crustacean is a new ectoparasite that could recruit its aquatic host only in the ocean. The two ectoparasite species found in this study were the only parasites undergoing a direct life cycle in which two host animals need to have bodily contact for transmission (chapter II & III). All other occurring parasite species use an indirect (heterogenous) life cycle with one or more obligate intermediate hosts. The stomach nematodes rely on preferred prey items of porpoises and seals as intermediate hosts for their transmission: E.g. herring transmits *Anisakis simplex* larvae infecting porpoises and cod is carrying *Pseudoterranova decipiens* larval stages infecting seals. New data gathered in this thesis showed that the highly pathogenic lungworms use several flatfish species in their life cycle. Flatfish are commonly consumed by both, porpoises and seals (Bjørge & Tolley 2002,

Gilles et al. 2008, Brasseur et al. in press), resulting in these prey items potentially carrying larvae of all six lungworm species infecting porpoises and seals in German waters. However, four of the investigated lungworm species infect only porpoises and two solely occur in seals. These strictly host-specific traits may result from the long common evolutionary history of these nematodes with their hosts.

A variety of factors mediate the compatibility of hosts and parasites, including ecology, physiology, immunity and genetics, which can vary between related species (Freeland 1983, Kennedy et al. 1986, Lile 1998, Whittington et al. 2000). Taxonomically related hosts are generally more prone to infections by the same parasite species spectrum (Thomas et al. 1995, Gregory et al. 1996, Bush et al. 2001). However, even closely related host species are not necessarily infected with the same species, prevalence and intensity or show the same pathology when infected by the same parasite species (Freeland 1983, Thomas et al. 1995, Lile 1998, Kapel et al. 2006). Tapeworm (Cestoda) infections in both porpoises and seals from German waters (Siebert et al. 2003, 2006b, 2008) occur regularly, although none were recorded in animals sampled for the studies presented in this thesis. The cestodes are seldom identified to species level (Herreras et al. 1997, Strauss et al. 1991) but may well belong to the same species (*Diphyllbothrium* sp.). They are believed to have first been restricted to marine birds and to have later colonised cetacean and then pinniped hosts during their radiation by 'host-switching' using the same prey species as intermediate hosts (Hoberg 1989, 1996). Severe lesions and high mortality due to infections with *O. circumlitus*, a lungworm infecting harbour seals in the Wadden Sea, in northern elephant seals (Gulland et al. 1997) suggest that this species is a poorly adapted host and might have been recruited recently.

No sex-related bias in parasitisation was observed between male and female host individuals in this study. In general, it is reported that parasite prevalence and intensity in mammals are higher in males (Poulin 1996, Zuk & McKean 1996, Klein 2000, Hudson et al. 2002, Moore & Wilson 2002). Biases in parasitism have been found to be due primarily to gender differences in the immune response. Hormonally mediated sex differences in susceptibility to parasitism exist for mammals, but statistically significant male biases in parasitism are not a general rule, especially not in helminth infections (Schalk & Forbes 1997).

The origin of the parasite samples from stranded, shot and bycaught porpoises and seals reflects a diverse range of animals, in which especially very young, very old and diseased

animals were included. The sampled individuals may not have been representative of a free-ranging population. It has to be assumed that some of the stranded specimens drifted in the ocean prior to stranding. Due to the unknown time period and the distance carcasses may have travelled, exact conclusions about the origin of these animals remain difficult. This is relevant especially for animals in a state of advanced decomposition for which e.g. the evaluation of the nutritional status is impossible. The applied statistical procedures controlled for this source of bias, and only animals in a good state of preservation (according to Siebert et al. 2001) were used for this study.

However, neither correlations of the nutritional status of the hosts with levels of parasitism nor seasonal patterns in parasite prevalences were detected. The analysis of data by age-class for all origins and both sexes of hosts indicated that age was the determining factor of infection, especially in harbour seals.

While larger sample sizes are accumulated over time periods longer than this study, more age-stratified data could be analysed for spatial and temporal patterns, as e.g. in chapter III in seals from around the two PDV epidemics.

A good nutritional status of the studied hosts should translate into better immune responses (Bundy & Golden 1987, Holmes 1993, Beisel 1996, Coop & Holmes 1996, Koski & Scott 2001) and lower parasite prevalences in the hosts. A weak negative relationship between infection with *O. circumlitus* and sternal blubber thickness was reported by Bergeron et al. (1997) in ringed seals from eastern Arctic Canada.

Most of the recovered parasite taxa in this thesis were transmitted by intermediate hosts and did not depend on physical contact between the animals. Therefore, the effect of group size and host density on the heterogeneity in parasite infection patterns has not been investigated. Harbour porpoises occur predominantly solitary or in small groups, like mother-calf pairs. The average group size in German waters was reported to be between 1.2 and 1.8 (Scheidat et al. 2004, Siebert et al. 2006a). Harbour seals are usually solitary when at sea, but haul out in groups of up to hundreds of animals (Herr et al. 2009). Future studies need to incorporate information of the parasitic life cycles, which should allow a more detailed view of the underlying transmission patterns. They should include the host's home range, which is especially important in the case of crustacean ectoparasites on the harbour porpoise. Climatic changes may drive new patterns in movements of top predators in the North Atlantic, following the migration or distributional shift of their prey. Consequently,

rare or new social interactions could occur and, as a consequence, new parasite-host relationships may develop (chapter II).

Multiple habitat characteristics are likely to interact, affecting parasite diversity. The habitat influence is of paramount importance when considering that almost all intestinal parasites release their propagules into the environment (Eckert 2000, Bush et al. 2001). Indirectly transmitted parasites depend heavily on favourable conditions for intermediate hosts to complete their life cycle (Lile 1998, Arneberg 2002). Given the scarcity of knowledge about potential intermediate and paratenic hosts, the diet composition of porpoises and seals (Gilles et al. 2008) and their habitat use, conclusions on the influence of different environmental and host–extrinsic factors can only be speculative. More research linking the diet and the migratory movements of the hosts will improve the validity of evidence on seasonal changes of parasite prevalences and intensities or enable a correlation of parasite loads with their nutritional status. This may also include possible prey selection processes by the two marine mammal species.

This study has shown that parasites can be used as valuable markers of contemporary and historical ecological relationships and evolutionary events of marine mammal taxa.

Despite potential sampling biases, parasites may prove useful to reveal information relevant for the conservation and management of marine mammal populations, particularly regarding stock identity, social structure and host movements (Balbuena et al. 1995).

(2) What are the consequences of parasitism for porpoises and seals?

Metastrongyloid nematodes from the respiratory tract played a major role in causing disease and mortality in harbour porpoise and harbour seal populations from German waters, in contrast to all other parasite species that were found not to be a major health threat to the animals (chapter I & III). Evaluating parasite pathogenicity is particularly difficult because the manifestation of disease symptoms depends on the interaction of many host- and parasite-intrinsic factors (Bush et al. 2001). Contaminants and other infectious agents (like Phocine Distemper Virus) that compromise the immune system may account for higher prevalences and severity of parasitic infections. Combined parasitological and pathological investigations proved to be a valuable tool in evaluating the health status and disease dynamics in wild animal populations. The necropsy findings and histology provided deeper insights into the

parasites' impact on the survival of wild hosts. The occurrence of lesions primarily caused by different parasite species and burdens reduced the function of the organs and affected their barrier effect against intruding pathogens and toxins. Lesions in the lung, ulcers in the stomach and scar tissue on the integument were often associated with secondary bacterial infections in the affected organs and led to serious illness and mortality. Thus, my results show that the infection with parasites frequently had a massive impact on the host's resource utilisation and the defence mechanisms in the different organ systems. However, the quantitative measurement of the number of parasites does not necessarily reflect the actual pathogenic potential of the parasite assemblage. The potential pathogenicity of each single parasite species and their interactive effects should be taken into account when assessing the outcome of the infection and the impact on the host's fitness. The observed simultaneous infections with several species in two or more organ systems likely affect the host stronger than an infection with only one species (chapters I, II & III). Infection with one highly virulent parasite species may have a more detrimental effect on the host than the infection with several other, less virulent parasite species. This was the case in parasite infections of the gastrointestinal tract in both harbour porpoises and harbour seals, in contrast to infections in the respiratory tract (chapter I & III). The effects of pollution and toxic contaminants have been shown to have immuno-suppressive consequences in marine mammals (Aguilar & Borrell 1995, Jepson et al. 1999, Siebert et al. 1999, Beineke et al. 2005) which might account for the higher impact of lung nematodes and associated lesions in porpoises from some geographic areas (chapter I). On the other hand, genetic differences between parasite populations could account for higher virulence of lungworms from the German North Sea (chapter I). However, the ITS-2 sequences of rDNA analysed in this study did not support this hypothesis. No intraspecific variation between ITS-2 sequences of lungworms from German and Norwegian waters was found (chapter IV). The investigation and comparison with additional markers from mitochondrial DNA would be helpful in future research.

The 1988/89 and 2002 Phocine Distemper Virus (PDV) epidemics, a pathogen known to effect the immune competence of seals (Ross 2002), significantly reduced the Wadden Sea population (Reijnders et al. 2005). During the epizootic and after recovering from it, harbour seals may have been more vulnerable for and susceptible to parasitic infections, especially in the respiratory tract, which is strongly affected by PDV. Because little data are available

about parasitism in seals from outside the epidemics, more research over longer time periods is necessary.

Comparisons with earlier data indicate that variation in parasitism can be due to temporal or spatial changes, methodology but probably also immunity or contaminant load of seals. Absence of lungworms in older seals may be due to mortality of severely infected juvenile animals, however, there is no evidence that infections only last a few months. Previously infected young seals may develop an acquired immunity and older unexposed seals may be resistant. Contaminants or other infectious agents that compromise the immune system may account for infections occasionally seen in older seals. Contrary to seals, young-of-the-year and neonate porpoises are generally not infected with pseudaliid lungworms. Prevalence increases with age (chapter I), suggesting horizontal transmission through the food web. The data also indicate that infections are accumulated with age and long-lived. This may be due to porpoise lungworms belonging to another family (Pseudaliidae) than seal lungworms (Crenosomatidae, Filaroididae). Nematodes are known as vectors of some viral, bacterial, and protozoan pathogens (Bird & Bird 1991) and lungworms have been shown to carry *Brucella* spp. (Garner et al. 1997) and calicivirus (Smith et al. 1980). Future research should investigate if lungworms can play a role in transmitting morbillivirus.

The sea represents a harsh environment for a warm-blooded mammal. Heat loss occurs much faster in water, food is often found only at depths, and dehydration is favoured by seawater. The equilibrium between physiological adaptations and environmental factors in marine mammal health is fragile and when it is disturbed, diseases may arise. Failures can be triggered by natural factors (e.g. food shortage and pathogens) or by human activities (e.g. pollution and habitat degradation), and it remains difficult to disentangle the origin of diseases in marine mammal populations.

(3) What can be gathered from the new data about lungworm ecology and how can they be used in future research?

This is the first study on lungworm ecology combining molecular methods, experimental infections of potential intermediate hosts as well as sampling and screening of possible intermediate hosts from the wild.

For the first time the ITS-2 (rDNA) sequences of seven metastrongyloid nematodes were recorded in this study. The ITS-2 sequences showed no intraspecific variation. As lungworms from porpoises originating from Norwegian waters were among the sampled individuals, it could be shown that there are no genetic differences between lungworms from different geographic regions, at least not in that marker. Clear interspecific differences within the ITS-2 of the investigated seven lungworm species were valuable for an unequivocal distinction between the species. The sequence data derived in this thesis are highly suitable for the identification of larval nematode stages when morphological characterisation is impossible. Additionally, the sequences were used for phylogenetic analyses to compare the relationship and origin of lungworms from marine hosts with those of terrestrial mammals.

Nematode larvae from possible intermediate hosts were isolated and most larvae from plaice were identified as *Parafilaroides gymnurus*, while larvae from dab consisted of several lungworm species. In general, dab were more frequently infected by larval nematode stages. It can be presumed that dab is an intermediate host for some of the lungworm species under study here, while plaice is more specifically used by *P. gymnurus*. There may be additional flatfish species like witch (*Glyptocephalus cynoglossus*) or lemon sole (*Microstomus kitt*) suitable as intermediate hosts for lungworm larvae.

A third flatfish intermediate host was identified using experimental infections. *O. circumlitus* represents the third lungworm species in this study that uses flatfish as intermediate hosts in their life cycle. No transport or paratenic host and/or invertebrate were involved in the transmission from first-stage larvae to the flatfish under experimental conditions. No previous study (Bergeron et al. 1997, Dailey 1970, Houde et al. 2003) that investigated lungworm life cycles in the ocean used molecular tools. Instead the identification of larval nematode stages was attempted using morphological characteristics or by criterion of exclusion. The location of live larval stages of lungworms in mucosa, mesentery and pancreas indicated immunological tolerance and the presence of larvae outside the intestine pointed to active somatic migration. The establishment of an *in-situ* hybridisation assay, as

provided by this study, proved to be a valuable tool to classify metastrongyloid nematodes from histological slides.

Adult female lungworms are ovoviviparous and release first-stage larvae in the airways, where they were observed in mucus. First-stage larvae were also observed in faeces. Presumably they are moved up the bronchial escalator by mucociliary action, swallowed into the stomach, passed through the gastrointestinal tract and excreted with faeces into the external environment.

Concluding from the results of this study, they sink to the sea bottom and, when taken in by flatfish, the larvae enter the intestinal wall and go through two moults in the mucosa, mesenteric tissue of the intestine or pancreas. Third-stage larvae retain cuticles of the first and second stages. When ingested by their mammalian final host, they probably develop into fourth-stage larvae in the stomach or intestine of the porpoise or seal, migrate to respiratory tract where they mature. Development within the definite host is unknown. Histological investigations routinely performed on organs of porpoises and seals (Siebert et al. 2008) commonly show multiple nematode sections in intestinal lymph nodes, liver and pulmonary lymph nodes in seals (and porpoises) infected with lungworms. It is likely that the sections show lungworm larvae in active somatic migration to the respiratory tract.

There may be an additional step in the life cycle during which larvae are ingested by invertebrates (ophiuroids, polychaetes) acting as transport hosts. The observed preferences for dab as intermediate host of *P. inflexus*, and for plaice in *P. gymnurus* remain unclear. Our data suggest that lungworms have their favourite intermediate fish hosts but further investigations should include more flatfish species to elucidate this phenomenon and to show which fish – if any – are used by the other three lungworm species.

This is the first study that identified intermediate hosts for certain lungworm species of marine mammals in the wild. For the first time, the transmission and indirect life cycle of two lungworm species of seals and porpoises in the Wadden Sea were documented. The newly developed molecular tools to identify larval lungworm stages may be used to diagnose parasites in live marine mammals from faecal samples and blowhole swabs. They will help define the life cycle of the lungworm species in more detail when tissue samples from dead stranded animals are screened and the somatic migration of larvae from the intestinal tract of the definite host to its respiratory tract is tracked (Lehnert et al. unpublished data). The development of a species-specific PCR and RFLP assay might be established for rapid species

identification of larvae and eggs of lungworms in the future. Additionally, molecular markers will be used as diagnostic tools to determine lungworm infection in various organs of intermediate and final hosts, e.g. in routine histological investigation of lung tissue slides of marine mammals.

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DESCRIPTION OF THE INDIVIDUAL SCIENTIFIC CONTRIBUTION TO THE MULTIPLE-AUTHOR PAPERS

The chapters of this thesis are published in international peer reviewed journals (Chapter I-III) or submitted to a peer reviewed journal (Chapter IV). The following list provides an overview about my personal contributions on each of the publications:

CHAPTER I: MACROPARASITES IN STRANDED AND BYCAUGHT HARBOUR PORPOISES FROM GERMAN AND NORWEGIAN WATERS

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published in: Diseases of Aquatic Organisms, 2005, Volume 64, 265-269

Contributions: US and KL developed the idea of this study. KL conducted the laboratory work and parasitological investigations, and was advised by and introduced to methods for identification of some parasite species by JAR. US evaluated the pathological findings. KL conducted the data analysis. KL, US and JAR discussed the results, KL wrote the manuscript. All authors read and approved the final manuscript.

CHAPTER II: *ISOCYAMUS DELPHINII*: FIRST RECORD OF WHALE-LICE ON A HARBOUR PORPOISE (*PHOCOENA PHOCOENA*) FROM GERMAN WATERS

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data analysis and documentation. KL wrote the manuscript. All authors read and approved the final manuscript.

CHAPTER III: PARASITES IN HARBOUR SEALS (*PHOCA VITULINA*) FROM THE GERMAN WADDEN SEA BETWEEN TWO PHOCINE DISTEMPER VIRUS EPIDEMICS

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CHAPTER IV: TRANSMISSION OF LUNGWORMS IN PORPOISES AND SEALS: MOLECULAR TOOLS DETERMINE VERTEBRATE INTERMEDIATE HOSTS

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Publikationen

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Gilles A, Herr H, **Lehnert K**, Scheidat M, Siebert U 2008. Harbour porpoises – abundance estimates and seasonal distribution patterns. In: Wollny-Goerke, K. and Eskildsen, K. (Eds.) *Marine mammals and seabirds in front of offshore wind energy*, Teubner, Wiesbaden, pp. 19-36

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ERKLÄRUNG

Hiermit erkläre ich, dass die vorliegende Dissertation selbstständig von mir angefertigt wurde und in Inhalt und Form meine eigene Arbeit ist. Es wurden - abgesehen von der wissenschaftlichen Beratung durch meine Betreuerin - keine anderen als die angegebenen Hilfsmittel und Quellen verwendet. Die Arbeit wurde unter Einhaltung guter wissenschaftlicher Praxis entwickelt.

Diese Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein erstes Promotionsverfahren. Die Promotion soll im Fach Zoologie erfolgen. Für die Prüfung wird die Form der Disputation gewählt. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

Kiel,

Kristina Lehnert