PATHOLOGY OF STRANDED MARINE MAMMALS

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Pathologie van gestrande zeezoogdieren

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GENERAL INTRODUCTION

MARINE MAMMALS: APPRECIATED, ELUSIVE AND INHABITANTS OF AN ENVIRONMENT UNDER PRESSURE

Life in the marine environment is under rapidly increasing pressure due to the effects of the exploding human population. Overfishing, chemical pollution, plastic waste, climate change and acidification, amongst others, have caused an alarming decrease of 49 % of population sizes in our oceans in the last four decades [1].

The fate of marine mammals in this tragedy is of interest as it can be instrumental in nature conservation. The first reason is that marine mammals are highly charismatic and can thus motivate actions by humanity which aim to preserve them. Examples are national and international treaties like the Marine Mammal Protection Act in the U.S., the Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Seas (ASCOBANS) in our region and the commercial whaling moratorium by the International Whaling Committee (IWC), which is a worldwide treaty. The second reason is that marine mammals are sentinels for the ecosystems they live in. They are particularly vulnerable to bycatch, chemical-, plastic- and noise- pollution, encroachment of critical habitat, and often compete with humans for the same food resources [2-8]. This means that entire ecosystems can benefit from the actions that are aimed at preserving marine mammal species.

Knowledge of viability and vulnerability of marine mammal populations is essential for choosing appropriate actions aimed at the conservation of marine mammal species. Such knowledge might be derived from analyzing data on population numbers and disease. Disease outcome is the result of the balance between pathogen, host, and environment [9]. The study of diseases may therefore provide information on environmental pressures like contaminants and food availability. The problem often is that data on population numbers and health are only limitedly available. The amount of data available largely depends on the accessibility of the species under investigation. Marine mammals are elusive, albeit some species more than others. Whales (including dolphins and porpoises) do not come on land when healthy, and differ in the amount of time they spend in areas where they are likely to be observed. Other marine mammal species do spend time on land, particularly when pupping, breeding and molting. This is reflected in our knowledge of population numbers and abundance trends. Of the 86 whale species, 25 are not threatened, 17 are threatened and 44 species are data deficient (meaning we have no idea about the viability of these species). For the other 41 marine mammal species (including eared seals, earless seals, polar bears, sea otters, walruses and sea cows) 24 are not threatened, 17 are threatened and no species are data deficient [10].

The information available for marine mammals commonly observed in the North Sea has a similar pattern. Most data are available for coastal living pinniped species like the harbor seal (*Phoca vitulina*), less data for coastal dwelling cetaceans like the harbor porpoise (*Phocoena phocoena*), and least data for pelagic living cetaceans like the Sowerby's beaked

whale (*Mesoplodon bidens*). Population numbers and trends are the most essential data for monitoring population viability. A look at what we know about these three marine mammal species, which are native to Dutch waters, gives the following information:
Harbor seal populations have been monitored by aerial surveys annually since 1960 by counting the number of molting animals and the number of pups hauled out on Dutch coasts [11]. These counts produce accurate numbers. However it is uncertain what part of the population is on land during the counting. A mark-recapture experiment determined the maximum likelihood number of harbor seals in the Dutch Wadden Sea in 1994 at 1536 with a 95 % confidence interval (CI) of 1 225 to 2 000 [12].
The harbor porpoise population in the same waters has been counted annually since 2011. Here uncertainty comes from the percentage of animals likely to have been observed. This depends on the sea state and turbidity of the water. Besides this uncertainty, the number counted on a line transect in an area has to be extrapolated for the area under investigation. This leads to very large confidence intervals e.g. in 2015 the 95 % CI was 21 194 - 79 256 [13].
For some elusive pelagic cetacean species there is no substantial knowledge at all. The International Union for Conservation of Nature (IUCN) states about Sowerby's beaked whales for example: "*There is no information on global abundance or trends in abundance for this species*" and "*Although it is one of the most commonly stranded Mesoplodon species, there have been few sightings at sea, and it is poorly known*." This species is, perhaps surprisingly, also native in Dutch waters [14].
For many marine mammal species, the quality of our protective measures is determined or limited by the quality of information we have. For example the ASCOBANS Conservation Plan for Harbor Porpoises (*Phocoena phocoena*) in the North Sea mentions that the main challenges in developing the conservation plan were a lack of data both on the target species and on the human activities and their actual or potential impact [15].

REHABILITATION CENTERS AS SOURCES OF INFORMATION ON POPULATION HEALTH

Animals in rehabilitation provide a highly significant opportunity for the study of marine mammal disease [16]. Phocine distemper virus, phocine herpesvirus and domoic acid intoxications of Californian sea lions were all discovered through or in close association with rehabilitation programs [17-19]. Several reasons for the success of the study of disease in rehabilitation centers can be mentioned. First, the level of observation and opportunities to sample are unique. Samples are fresh and, while the animal is alive, sequential sampling can be done. This increases the chance to discover agents that quickly disappear post mortem through autolysis, and to culture agents that cannot maintain themselves in dead cells. Furthermore, observations done by pathologists can

be related to clinical observations. This helps to establish the relation between observed pathology and clinical signs and puts the pathologic observations into context. It is rarely possible to establish the relation between clinical and pathological observations in free-living wildlife.

However, there are also limitations to the study of disease in rehabilitation centers. The most relevant limitation is that animals that become available through stranding and rehabilitation programs are not representative for the population in the wild. Dolphins and porpoises mostly strand because they are ill or undernourished. Furthermore, animals that strand likely reside in the neighborhood of the coast. The composition of the population near the coast may well be different from the entire population in age and gender. Finally, diseases that lead to a very quick death will not be observed in rehabilitation centers. Bycatch is the most important and obvious example. Together, these limitations restrict the possibility to extrapolate information gathered in rehabilitation centers to the health of the population in the wild [16]. Stranded and rehabilitated animals may serve rather as sentinels or indicators of disease or abnormalities than as representatives for their wild counterparts [20].

PATHOLOGY OF HARBOR PORPOISES

Research into pathology of harbor porpoises is important as the species occurs in regions heavily influenced by human activities and large, poorly understood, population fluctuations have been observed [21]. The harbor porpoise was abundant in Dutch waters up to the Second World War, but the population likely declined shortly thereafter. In the sixties, seventies and eighties of the last century, the species was virtually absent in Dutch waters. The observed population decline was not only a concern in the Netherlands but also in Belgium [22], Germany [23] and the United Kingdom [24]. Reasons for its disappearance are poorly understood. Common seals also virtually disappeared in this period. For this species, pollution with polychlorinated biphenyls (PCBs) was speculated to be one of the causes of the decrease in population in the Wadden sea and the Scheldt estuary [25]. Harbor porpoise were most likely exposed to similar pollution. Large-scale studies into the pathology of harbor porpoises have been done between 1980 and 2000, mainly in the North East Atlantic with a special focus on the North Sea and adjacent waters. These studies can be divided into studies on stranded animals [22, 24, 26, 27], by-caught or hunted animals [28-30], or a mix thereof [23]. Further research has focused on specific aspects of the pathology of harbor porpoises, namely helminth parasites [31, 32] and pulmonary pathology [33], based on animals from the general studies mentioned above.

The top three (probable) causes of death in research on stranded harbor porpoises were drowning due to bycatch, bronchopneumonia (bacterial and/or parasitic) and emaciation (mainly of neonates). In 7 to 25 % of animals, cause of death could not be determined.

INFECTIOUS PATHOGENS OF SMALL ODONTOCETES

Advances in research of viruses in marine mammals were made possible by developments in diagnostic techniques from the last two decades of the 20th century onward. The 1989 large scale epidemic of morbillivirus in common seals in North Western Europe provided additional motivation to explore this research topic. Since 1980 representatives of eleven virus families have been observed for the first time in cetacean species (*Pesti-, Pox-*, *Papilloma-*, *Adeno-*, *Herpes-*, *Orthomyxo-*, *Paramyxo-*, *Hepadna-*, *Retro-*, *Rhabdo-* and *Caliciviridae*) [34-44]. For many viruses, it remained uncertain if they are capable of causing disease and/or death. In case of influenza-, parainfluenza-, hepadna- and adeno- viruses, strong indications are present that these viruses can cause disease in cetaceans [36, 38, 40, 45]. Herpesvirus and morbillivirus can cause death in cetaceans, with morbillivirus being the only virus documented to have caused large-scale epidemics with significant fatality rates [46, 47].

Pestivirus

Pestiviruses are a genus of *Flaviviridae*. They are mostly known for their ability to cause disease in livestock and their consequential large financial impact. Examples are bovine virus diarrhea virus in cattle and classical swine fever virus in pigs. Pathogenesis is dependent upon characteristics of the pestivirus strain (biotype and virulence), age at which infection occurs, and host characteristics (comorbidities and immune status). Two biotypes of pestivirus are defined according to the ability of the virus to cause a cytopathic effect (cell lysis) in tissue cultures: non-cytopathogenic (ncp) pestiviruses and cytopathogenic (cp) pestiviruses [48].

As pestiviruses are capable to cross the placenta, fetal infection occurs. This may lead to abortion, mummification, the birth of non-viable offspring or a persistent infection (PI). Fetal infection can lead to PI if infection occurs before the immune system of the fetus has matured and infection is by a ncp pestivirus. The immune system classifies the pestivirus as "self", in such a case, which results in immunotolerance, a PI of virtually all organ systems, and a continuous shedding of the virus [49]. PI animals are, at least in bovines, important to maintain the infection in a population [50]. PI cattle may develop and die of mucosal disease, which results when a coinfection of the ncp-virus-infected animal with cp virus occurs. In swine, late onset classical swine fever may occur in PI piglets [48].

Disease outcome varies tremendously between infected juvenile and adult animals. Infections can be acute to chronic and lead to mortality or be without any signs of disease [48]. Signs of disease can be the result of direct cytopathic effect of the virus on infected organs or be the result of immunosuppression as the virus targets certain components of the immune system [48, 50].

Evidence of pestivirus infection has been observed in Cervidae, Bovidae, Suidae and

Leporidae. Pestiviruses are not strictly host specific [48]. With the arrival of metagenomics, putative infections with pestiviruses have been observed in other mammalian families: in brown rats (*Rattus norvegicus*) of the family Muridae and intermediate horseshoe bats (*Rhinolophus affinis*) of the family Rhinolophidae [51, 52]. It is unknown if these pestivirus infections in wild animals have a significant effect at a population level with the exception of the infection of Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). Populations of Pyrenean chamois were decimated by pestivirus outbreaks [48, 53].

Herpesvirus

Herpesviruses are ubiquitous viruses with at least one representative found in each mammalian species investigated. A hallmark of the order *Herpesvirales* is their life long, often latent, persistence in infected hosts.

The family of *Herpesviridae* has three subfamilies, each with its own target cells for latent virus: *Alphaherpesvirinae* (latent in neurons), *Betaherpesvirinae* (latent in monocytes and T-lymphocytes) and *Gammaherpesvirinae* (latent in B-lymphocytes) [54].

Reactivation of latent virus and the nature of an active infection is largely related to the immune status of the host. Herpesviruses have co-evolved with their hosts and usually have a narrow host range. In their natural hosts they usually cause temporary symptoms or disease if the host is immunocompetent. At the extremes of age, in immunocompromised individuals or in individuals of species closely related to their natural host species, infections can be more severe and lethal [55].

Herpesvirus-associated lesions in cetaceans range from mucosal and/or cutaneous lesions [56-58] to encephalitis [59-61] and disseminated systemic disease [46]. Gammaherpesviruses have been associated with mucosal lesions [57] and cutaneous lesions [57, 58]. Alphaherpesviriuses have been associated with cutaneous lesions as well as encephalitis and disseminated systemic disease [46, 57, 59, 60]. In harbor porpoises, the only reported herpesvirus infection is a case of encephalitis associated with herpesviral antigen expression in affected neurons [60], and several cases of dermatitis, in which a gammaherpesvirus, tentatively named Phocoenid herpesvirus-1, was detected by PCR [58].

Morbillivirus

Morbillivirus is a genus of viruses belonging to the family of *Paramyxoviridae* which is part of the order *Mononegavirales.* As the name suggests this order of viruses has a single RNA strand of negative polarity. The genus *Morbillivirus* currently holds seven species, three of which are known etiologic agents of large scale epidemics in marine mammals: *Canine distemper virus* (CDV), *Phocine distemper virus* (PDV) and *Cetacean morbillivirus* (CeMV) [62]. Morbilliviruses are highly contagious viruses and frequently cause 'virgin soil' epidemics in naïve marine mammal populations. In 1988 and 2002, PDV decimated the harbor seal

population in northwestern European coastal waters [63, 64]. Measles in humans, caused by the human morbillivirus (measles virus), does not invoke latent carriers who at a later stage shed the virus, as happens in herpesvirus infections. Measles virus therefore needs a population between 200 000 and 500 000 individuals to remain endemic, depending on population density [65]. For animal morbilliviruses a similar epidemiology is assumed [66]. This implies that for every epidemic observed in relative small or fragmented population there should be a larger endemically infected population of the same or other susceptible species, as a source of spill-over infection.

CeMV virulence appears to differ with host species and immune status. CeMV has caused epidemics with high mortalities in bottlenose dolphins (*Tursiops truncatus*), common dolphins (*Delphinus delphis*), long-finned pilot whales (*Globicephala melas*), and striped dolphins (*Stenella coeruleoalba*) [47, 67-70]. These epidemics are the result of the introduction of the virus into a population that is largely unprotected due to lack of antibodies as a consequence of lack of previous exposure and resulting lack of immmunity. In none of these epidemics a source population of infection has been identified.

In eight species of odontocetes, exposure to morbilliviruses has been observed either by detecting antibody in sera (Risso's dolphin [*Grampus griseus*], Atlantic white-sided dolphins [*Lagenorhynchus acutus*], Fraser's dolphins [*Lagenodelphis hosei*], Atlantic spotted dolphins [*Stenella frontalis*], and false killer whales [*Pseudorca crassidens*]) [71] or by sporadic pathogen detection (harbor porpoises [72], white-beaked dolphins [73, 74], and a pygmy sperm whale [*Kogia breviceps*]) [75]. The virulence of morbillivirus infection for these latter eight species is unclear.

Morbillivirus infection has two ways of causing disease. First the virus targets lymphotropic tissues, which may cause severe immunosuppression and thereby facilitate or exacerbate pre-existing or secondary infections. Second the virus may cause direct damage to infected organs. Virus infection of the brain may be associated with inflammation, necrosis and demyelinization [76].

Chemical pollution, with PCBs and dichlorodiphenyltrichloroethane (DDT), has been associated with enhanced mortality in outbreaks of morbillivirus in the 1988 epidemic in harbor seals in the North Sea and in the 1990 outbreak in striped dolphins in the Mediterranean [77, 78].

Morbillivirus transmission occurs between marine mammal species and across the terrestrial-aquatic interface. Transmission dynamics are complicated and poorly understood. For PDV it is unknown where or from what species the large scale infection of harbor seals originates, and why gray seals are less susceptible. For CeMV it is unknown what the differences of susceptibility to CeMV infection are between cetacean species and to what extent different strains transmit between members of a single cetacean species or if virus spread is sustained by a multi-host transmission cycle, as is observed for CDV[79].

Staphylococcus aureus

Staphylococcus aureus is an important pathogen for most warm-blooded animals, including aquatic mammals. *S. aureus* lives as a commensal on skin and mucous membranes of warm-blooded animals. Approximately 50 % of humans are carriers [80]. As an opportunistic invader, it is an important pathogen for humans and other animals. In humans it may cause septicemia, endocarditis, pneumonia as well as wound, bone, and joint infections [81]. Similar diseases occur in other animals [82]. In marine mammals, *S. aureus* has been identified as one of the most important pathogens in bottlenose dolphins under human care and in pinnipeds admitted into a rehabilitation center [83, 84]. In stranded harbor porpoises, *S. aureus* has been observed as a cause of abscessation, pneumonia and septicemia [22, 23]. In the two collections of harbor porpoises held in zoos in Europe, two out of eight animals died with *S. aureus* infection of the myocardium. The question which imposed itself is what the origin of *S. aureus* infection in these harbor porpoises was and in a broader sense what the origin of *S. aureus* infections in marine mammals is.

Different *S. aureus* isolates or lineages are associated with different host species. However they can transmit from one species to another and this has been documented from humans to animals and vice versa [85, 86]. Genetic analysis of the different isolates provides information on origin and spread between animal species by *S. aureus* isolates. The genetic analysis of *S. aureus* isolates uses Multi Locus Sequence Typing.

Multi Locus Sequence Typing sequences 7 housekeeping genes. Housekeeping genes are constitutive genes (expressed constantly) that have a relatively slow rate of change. This makes them particularly appropriate for studying larger scale population dynamics. Approximately 450 base pairs are sequenced for each gene and as each gene has between 11 and 17 different alleles it is highly unlikely different *S. aureus* isolates have the same allele combination by chance. Each isolate is assigned a Sequence Type (ST) based on the allele combination observed. Similar STs are a clone and a Clonal Complex (CC) consists of the STs which are identical on 6 out of 7 household genes. For CCs it is assumed they have originated from a common ancestor [87]. In human medicine this technique is useful for figuring out where methicillin-resistant *Staphylococcus aureus* (MRSA) isolates originate from and how they spread. We could use this technique to investigate if marine mammals, which are uniquely warm-blooded animals (and therefore unique potential carriers of *S. aureus*) in the marine environment, have their own *S. aureus* isolates or if they contracted *S. aureus* isolates through contact with terrestrial species.

Lungworms

Harbor porpoises in the North Sea are commonly infected by four species of nematodes belonging to the family of the Pseudaliidae: *Halocercus invaginatus, Pseudalius inflexus, Torynurus convolutus* and *Stenurus minor* [22, 32]. The sizes of adult nematodes in the lungs

vary from 15 cm length for female *P. inflexus* to 18 mm length for male *S. minor*. Prevalence and intensity observed by various researchers varies from 22 % to 100 % with intensities varying from 20 (*P. inflexus*) to 2362 (*S. minor*) [32]. With the exception of *H. invaginatus,* pathogenesis is assumed to involve infection by a heteroxenous host of larval worms. Infection of neonate harbor porpoises is seen with *H. invaginatus* only, suggesting trans-mammary or in utero infection [88]. Infection with the other three species only occurs in juveniles with a minimum age of 7 months [33].

Nematode infections of the lungs of harbor porpoises have been held responsible for a large percentage of mortalities of stranded harbour porpoises. For example. Siebert et al. concluded parasitic bronchopneumonia's were responsible for two thirds of the mortalities of 133 necropsied harbour porpoises [23], while Jauniaux et al. considered 27 out of 55 animals to have died due to '*severe and extended parasitosis*' [22]. These mortalities were either attributed to occlusion of the airways by parasites or to inflammation, at times aggravated by a bacterial or fungal infection.

Researchers have given different points of view about the consequences of high intensity infections with lungworms. Baker et al., Siebert et al. and Jauniaux et al. state porpoise deaths could be attributed to high intensity infections blocking the respiratory airways [23, 26, 33]. Kirkwood et al. and Clausen et al. state that harbor porpoises are able to tolerate large burdens of lungworms and that high intensity infections did not correlate with poor nutritional condition or low blubber mass in bycaught animals [24, 28]. Several researchers suggest bacterial pneumonias are a consequence of lungworm infection and are solely observed in conjunction with lungworm infections [22, 23, 33].

OUTLINE OF THIS THESIS

The goal of my research was to investigate causes of morbidity and mortality in marine mammals with special attention for the impact of human activities. Accurate knowledge on prevalence, incidence and virulence of infectious diseases and other causes of morbidity and mortality is instrumental in the assessment of the vulnerability of marine mammal species and in the selection of mitigating actions that help the conservation of these species and their ecosystems. Furthermore this knowledge provides a benchmark for monitoring changes in causes of morbidity and mortality over time and space. Changes in morbidity and mortality can only be related to changes in the host, disease agent or environment if this benchmark knowledge is available. The knowledge needs to be detailed as changes might be subtle.

The foundation of this thesis is based upon autopsies performed on stranded small cetaceans which died in the rehabilitation center SOS Dolfijn in Harderwijk between 2003 and 2016. Most animals offered for autopsy were harbor porpoises. For this species numbers of investigated animals allowed to obtain a relevant insight in the pathology

encountered in this species upon stranding.
Autopsy and sampling were done according to protocol [89] as was the reporting. Gross pathology and histology were consistently performed by a board-certified pathologist (professor Kuiken) assisted by myself as an experienced aquatic mammal veterinarian. Material for investigation was mostly collected fresh, which in combination with the observed pathology and clinical signs observed while alive, adds to the unique nature of the data generated as described in **Chapter 2** of this thesis.
I chose to investigate five important pathogens of marine mammals which were encountered during autopsies; herpesvirus, morbillivirus, pestivirus, lungworms and *S. aureus*.
In three harbor porpoises, clinical signs of encephalitis were observed during rehabilitation. Encephalitis was diagnosed by histology after their death. No clear etiology was observed for any of these cases and samples were submitted for next generation sequencing in an attempt to find the infectious agent responsible for the encephalitis. Although no etiology for the observed encephalitis was found, we did find a novel pestivirus in our archived material. **Chapter 3** describes the search for hitherto undiscovered viruses in archived samples of dead harbor porpoises.
Observations during autopsies of stranded animals offered the opportunity for further and deeper investigations into viral diseases of odontocetes. The setting within the Department of Viroscience of Erasmus MC provided a welcome opportunity for this, especially as very little is known about viral diseases in odontocetes. Intranuclear inclusion bodies (INIB) were observed associated with encephalitis in one harbor porpoise and in a genital plaque in another harbor porpoise. The INIB in both lesions turned out to be filled with herpesvirus-like particles. This was the starting point to investigate the pathology and epidemiology of herpesviruses in harbor porpoises. Concurrently I investigated the origin of genital plaques in a collection of bottlenose dolphins under human care. The herpesvirus associated with these genital plaques was successfully propagated *in vitro*. This facilitated the development of an ELISA to detect specific antibodies and investigate the epidemiology of this infection by looking at antibody titers in archived serum samples. **Chapter 4 and 5** describe the results of my investigations into herpesvirus infections in harbor porpoises and bottlenose dolphins, respectively.
Two white-beaked dolphins, infected with morbillivirus, were admitted into the rehabilitation centre SOS Dolfijn. Observations on the live animals together with observations of gross autopsy and histology made a cautious analysis of the virulence of morbillivirus in this species possible. **Chapter 6** describes the results of my investigations into morbillivirus infection in white-beaked dolphins.
A harbor porpoise of the zoological collection of non-releasable rehabilitated animals died due to a *S. aureus* myocarditis with abscesses in the heart muscle. This harbor porpoise was the second harbor porpoise in human care which was shown to have died of such an infection in Europe. Given the very small numbers of harbor porpoises held under human care it was at least remarkable. Relevant questions are whether humans could have been

the source of these *S. aureus* infections, whether marine mammals (gray seal, southern elephant seal, harbor seal and harbor porpoise; see box 1 below) harbour their own host species specific *S. aureus* isolates or whether these animals were contaminated by *S. aureus* isolates originating from terrestrial animals. I looked at infections with *S. aureus* in harbor porpoises, harbor seals, a gray seal and an elephant seal (for more information on these species see box 1). **Chapter 7** describes my investigation into this topic.

Chapter 8 is a summarizing discussion which weighs my findings in the context of existing knowledge, with some thoughts on future research.

BOX 1: ***A SHORT INTRODUCTION OF THE MARINE MAMMAL SPECIES ENCOUNTERED IN THIS THESIS***

The harbor porpoise (*Phocoena phocoena)*

The harbor porpoise is a small cetacean that inhabits mostly coastal waters of temperate and sub polar zones in the Northern Hemisphere. Females are larger than males with maximum sizes of 190 and 140 cm, respectively. Global population is estimated at 700 000 animals [90], half of which swim in the North Sea [91]. Approximately 41 000 animals reside in Dutch waters [13]. Prey are mostly very small fish between 3 and 10 cm long. Recent research indicates porpoises may feed day and night and catch up to 550 fish per hour [92]. Harbor porpoises themselves are eaten by killer whales, sharks and grey seals [93]. Bottlenose dolphins kill harbor porpoises but do not eat them [94]. Harbor porpoises are mostly solitary. Sexual maturity is reached at 3 to 4 years of age, after which females may calve every one to two years. Sexual behavior is promiscuous, with males having large testicles indicating sperm competition for reproductive selection [90].

The conservation status for the global population is of least concern [95]. For Dutch waters, the conservation status is unfavorable [96]. Threats to the population in Dutch waters which are a consequence of human activity are bycatch, and pollution (mining, windmill construction and exploitation, marine litter, acoustic and chemical pollution a.o.). Infectious diseases are natural threats but their impact on health may become more severe when human activities decrease immune resistance and or nutritional condition. Research is needed for a proper quantification of these threats [21].

The white-beaked dolphin (*Lagenorhynchus albirostris*)

The white-beaked dolphin is a medium-sized dolphin which inhabits cold temperate to subpolar waters of the North Atlantic. Adult males are larger than females and can reach to 310 cm long and weigh 350 kg. Global population estimates exceed 100 000 [97], with approximately 20 000 animals residing in the North Sea [91]. They are found along the continental shelf and feed upon demersal, small schooling fish, squid and crustaceans. Killer whales have been observed to hunt white-beaked dolphins [98]. White-beaked dolphins are social animals and live mostly in groups of ten animals or less [99]. Little is known about reproductive behavior but it appears to be seasonal [100]. The global conservation status is classified as of least concern [97]. Nevertheless, some scientists are concerned about the negative influences that bycatch, displacement due to climate change, noise pollution and chemical pollution may have [101-104].

The bottlenose dolphin (*Tursiops truncatus*)

Bottlenose dolphins are medium-sized dolphins who can be found in nearly all temperate and tropical marine waters. They are mostly coastal dwellers. Typically adults are 250 cm long and weigh between 200 and 300 kg. Males are slightly larger than females. Global population is at least 600 000 animals [105]. Their diet consists of fish and squid [106]. Large sharks prey on bottlenose dolphins[107]. They are social animals and tend to live in groups of 2 to 15 individuals, best characterized as fission-fusion societies [108, 109]. Sexual maturity is reached in females between 5 and 13 years of age and in males between 10 and 13 years of age [110, 111]. Females have spontaneous sporadic seasonally influenced ovulations and calve on average every 3 years. Calves remain with their mothers for 3 to 6 years [112]. Males may visit groups of females alone or as pairs for reproductive purposes and will bond with females for periods varying from minutes to weeks. The mating system is thus a polygynandry (both males and females have multiple partners) [113].

The conservation status at a global level is of least concern. However, local populations can be under serious pressure. Significant threats include: catch and bycatch, chemical pollution, reduced prey availability due to habitat degradation and overfishing, direct and indirect disturbance and harassment, marine construction and demolition and other forms of habitat destruction and degradation [105]. Local populations have been decimated by morbillivirus epidemics. The impact of these epidemics may have been aggravated by chemical pollution. [47, 114, 115].

The harbor seal (*Phoca vitulina*)

The harbor seal is a true seal, earless and crawling. Harbor seals live in coastal temperate and polar waters of the Northern hemisphere. Males are slightly larger than females, adults reaching sizes of 160 to 190 cm long at 70 to 150 kg weight. Harbor seals have a high site fidelity and haul out mostly on a daily basis. Global population is around 600 000 animals [116]. Approximately 8 500 animals reside in Dutch coastal waters [117]. This is a large increase from the lowest population measured in 1976, 495 animals, and after the 1988 morbillivirus epidemic, 550 animals. Harbor seals are generalist opportunistic feeders that take a wide variety of fish, cephalopods and crustaceans, depending upon location and season [118-121]. Seals are predated upon by killer whales, sharks, walruses, Steller sea lions, eagles, gulls, and ravens [122]. Their social organization is not very clear. Although mostly reported as solitary [123, 124], they do exhibit play, contact and dominance behavior as well as group formation on haul-out grounds, which indicates a social network is important for seals [125]. Male competition for females occurs and this leads to a polygyny (males have multiple females) system of mating [126]. Female seals become sexually mature between 3 and 5 years and males between 4 and 6 years old. They are seasonal breeders and pups are weaned at 26 days of age. A month after weaning mothers ovulate and mate [116].

Conservation status is of least concern at a global level. Major threats are mass die-offs due to viral outbreaks (i.e. morbillivirus epidemics). Exposure to terrestrial animals and humans and their waste create risks of communicable diseases and chemical pollution. Noise pollution may affect foraging behavior and physical condition. Fishing may lead to bycatch and prey depletion. Environmental changes (e.g. climate change) is likely to cause changes in prey availability [116].

The southern elephant seal (*Mirounga leonina*)

Southern elephant seals are the largest pinnipeds and have the most extreme sexual dimorphism. Adult males grow to 450 cm long and weigh 2 to 4 tons, females grow to 280 cm long at weights of 400 to 900 kg. The species inhabits the entire Southern Ocean. Global population was last estimated at 650 000 animals in the mid-nineties of the last century and population size has increased during the last three generations (28.5 years) [127]. Elephant seals live most of their time at sea and may travel 5 000 km from their terrestrial haul-out sites. In fact, adult elephant seals spend over 65 % of their time below 100 meters [128]. Their diet consists of fish (including lantern fishes) and squid [129, 130]. They migrate biannually to haul-out sites in order to breed and moult. Apart from their interactions at haul-out sites, little is documented on their social behavior. Southern elephant seal females mature at ages 3 to 6 years old and males at 4 and 5 years old. Females pup a week after hauling out and subsequently lactate for three weeks before they set out to sea again. Shortly before weaning they mate. Bulls arrive before the females at the rookeries and set up a hierarchy. Beachmasters (dominant bulls) will almost exclusively mate with all females in their breeding aggregation, although sometimes they have to allow assistant beachmasters or possibly sneak copulation by subordinate males. The mating system is thus a polygyny [131].

Conservation status is of least concern. Threats are few due to this species living in remote areas undisturbed by humans and their actions. The influence of climate change is uncertain [132, 133].

The gray seal (*Halichoerus grypus*)

Gray seals are large sexually dimorphic, true seals. In the eastern Atlantic, males grow on average 200 cm long and weigh 233 kg, females 180 cm and 155 kg. They live in cold temperate to sub-Arctic waters in the North Atlantic Ocean [134]. Although gray seals are mostly coastal and forage in the vicinity of haul-out sites, they can travel occasionally for several thousands of kilometers to other known haul-out sites [135]. Global population is around 630 000 animals and increasing [136]. In Dutch waters, gray seals returned in 1978 after an absence of centuries [137]. At present, around 5 300 animals live in Dutch waters. Gray seals are generalist feeders. They prey mostly on benthic and demersal species and in Dutch waters sporadically on harbor porpoises [138]. Gray seals are predated upon by killer whales and sharks [139, 140]. They are solitary animals with most social interactions during breeding time. Female gray seals become sexually mature between 3 and 5 years of age and males at 3 years of age but rarely mate before they are 8 years old. Females bear their young, lactate and mate during an 18-day stay in the breeding colony. Dominant males reside up to 56 days in a breeding colony where they attempt to mate with as many females as possible. The mating system is thus polygynous. However, research does indicate not all seemingly dominant males are the parent of the offspring of the females under their guard, possibly due to sneak copulations in the water or at the periphery of the colony by subordinate males [141]. Conservation status is of least concern at global and population levels [136]. Major threats are bycatch in fishing nets and population control measures to protect commercially important coastal fisheries and chemical pollution, the last most notably in the Baltic [142].

harbour porpoise, Danish coast
Jonas Teilmann

2

CLINICAL, PATHOLOGICAL, AND LABORATORY DIAGNOSES OF DISEASES OF HARBOR PORPOISES *(PHOCOENA PHOCOENA)*, LIVE STRANDED ON THE DUTCH AND ADJACENT COASTS FROM 2003 TO 2016.

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ABSTRACT

Harbor porpoises (*Phocoena phocoena*) in the North Sea live in an environment heavily impacted by humans, the consequences of which are a concern for their health. Autopsies carried out on stranded harbor porpoises provide an opportunity to assess health problems in this species. We performed 61 autopsies on live-stranded harbor porpoises, which died following admission to a rehabilitation centre between 2003 and 2016. The animals had stranded on the Dutch (n=52) and adjacent coasts of Belgium (n=2) and Germany (n=7). We assigned probable causes for stranding based on clinical and pathological criteria. Cause of stranding was associated in the majority of cases with pathologies in multiple organs (n=29) compared to animals with pathologies in a single organ (n=18). Our results show that the three most probable causes of stranding were pneumonia (n=35), separation of calves from their mother (n=10), and aspergillosis (n=9). Pneumonia as a consequence of pulmonary nematode infection occurred in 19 animals. Pneumonia was significantly associated with infection with *Pseudalius inflexus, Halocercus sp.,* and *Torynurus convolutus* but not with *Stenurus minor* infection. Half of the bacterial pneumonias (6/12) could not be associated with nematode infection. Conclusions from this study are that aspergillosis is an important probable cause for stranding, while parasitic infection is not a necessary prerequisite for bacterial pneumonia, and approximately half of the animals (29/61) stranded probably due to multiple causes. An important implication of the observed high prevalence of aspergillosis is that these harbor porpoises suffered from reduced immunocompetence.

INTRODUCTION

Biodiversity is in sharp decline due to increasing human pressures on the environment. The marine environment is no exception and vertebrate population abundance loss in the oceans has been estimated at 36 % between 1970 and 2012 [143]. Therefore, there is justifiable concern for the conservation of marine species and ecosystems in areas where humans have a large impact. This includes the harbor porpoise (*Phocoena phocoena*) living in the North Sea, an environment heavily influenced by human activities.

Anthropogenic activities in the North Sea lead to chemical pollution [5], noise pollution [4], and depleted fish populations [3], which all may affect harbor porpoises. Firstly, they are vulnerable to chemical pollution because they bioaccumulate and biomagnify lipophilic chemical pollutants [5]. Multiple investigations have found indications for the negative effect of these chemical pollutants on the immune system of harbor porpoises in the North Sea and adjacent waters [30, 144-146]. Secondly, harbor porpoises are vulnerable to noise pollution because their hunting and communication are largely dependent on acoustic signals. Thirdly, they are vulnerable to fishing activities because they drown due to accidental capture in fishing gear [147] and because harbor porpoises partly depend on fish species that are also targeted by human fisheries [148].

Historic observations on the abundance of harbor porpoises in the North Sea suggest it is a vulnerable population. Harbor porpoises were abundant in Dutch coastal waters until the early fifties of the last century, went nearly extinct in the seventies and eighties, but showed a strong population increase in the decades thereafter [149]. The reasons for these fluctuations in abundance are largely unknown, although chemical pollution and fisheries bycatch have been implicated as causes for the population decline[150].

Previous investigations, among harbor porpoises stranded and bycaught around the North Sea between 1990 and 2000, have shown that the top three (probable) causes of mortality are bycatch, bronchopneumonia (bacterial, parasitic or a combination of the two) and starvation (mainly of neonates) [22-24, 26].

It is unknown, however, whether causes of mortality have changed since 2000 or if these causes of mortality were different around the Dutch coast compared to those other regions of the North Sea. Moreover, there is no consensus on the impact of parasitic lung infections on the health of harbor porpoises. Some researchers regard pulmonary parasitic infections as a primary cause of death [23, 24, 26], or as the trigger for secondary and lethal bacterial pneumonias [22, 23], while others have observed heavy infections without apparent health effects [24, 28].

Our goal, therefore, was to establish the probable causes for stranding of harbor porpoises around the Dutch coast in comparison with previous surveys [22-24, 26], and to evaluate the role of parasitic lung infections as a cause of pneumonia.

Autopsies were performed on harbor porpoises that stranded alive on the coasts of the Netherlands or neighbouring countries between 2003 and 2016, were rescued, but despite

rehabilitation efforts died or had to be euthanized, while in captivity. The advantage of this set up was that we had clinical and pathological data of these animals, and that carcasses were always fresh.

Our main findings showed that cause of stranding was associated mostly with alterations in multiple organs (n=29) rather than alterations in a single organ (n=18). Nematode infections resulted in pneumonia in 19 animals and was significantly associated with infection with *Pseudalius inflexus*, *Halocercus sp.*, and *Torynurus convolutus* but not with infection with *Stenurus minor*. Half of the bacterial pneumonias (6/12) occurred independently of nematode infection. We observed aspergillosis in an unprecedented high prevalence 14.7 % (n=61). These results suggest the immunocompetence of our sample of harbor porpoises was reduced compared to the samples of harbor porpoises in previous surveys [22-24, 26].

MATERIALS AND METHODS

Rescue and rehabilitation of live-stranded cetaceans at SOS Dolfijn

Since 1967, small cetaceans—mainly harbor porpoises—that strand alive along the Dutch, Belgian and German coasts have been rescued and rehabilitated at the Dolfinarium Harderwijk (Harderwijk, The Netherlands) and subsequently released into the wild. Since 2004, this activity was operated by an independent foundation, SOS Dolfijn, at the same site. Admission and rehabilitation of live stranded wild harbor porpoises at the SOS Dolphin Foundation was authorized by the government of the Netherlands (permit number FF/75/2012/036). SOS Dolfijn had two 50 m^3 pools with fresh water to which sodium chloride was added. In the first period of rehabilitation, animals were observed round the clock and standard parameters were recorded, including respiration rate, cramps, food intake and defaecation. In addition, other potentially relevant observations were recorded, including swimming behaviour and alertness. As an animal improved, the level of observation and care diminished to a minimum of 9 hrs per day.

Age determination of autopsied harbor porpoises

Age classes were defined according to the following criteria [151]: neonates, animals less than a week old based on remains of umbilicus or time of year found (June, July), body weight up to 11 kg and body length up to 90 cm; juveniles, immature gonads (testis weight < 100 g each for males; absence of corpus luteum or corpus albicans on ovaries for females) and body length < 130 cm for males and < 145 cm for females; adults, mature gonads (testis weight > 100 g each for males and presence of corpus luteum, corpus albicans or follicle > 1 cm diameter for females) or with a body length > 130 cm for males or > 145 cm for females. Ages of juveniles were estimated by comparing length at admission with published age length data [151] and assumed date of birth on the first of July [152].

Autopsy and histology

Autopsies were performed according to a standard protocol [89], and by the same pathologists. The following tissues were sampled for histology: adrenal gland, bronchus, cerebellum, cerebrum, colon, duodenum, oesophagus, forestomach, fundic stomach, gonads, heart, jejunum, kidney, liver, lung, mesenteric lymph node, muscle, pancreas, pulmonary lymph node, pyloric stomach, skin, spleen, thymus, thyroid, trachea, tracheobronchial lymph node, and urinary bladder. Additional samples were taken of tissues with gross lesions. Tissue samples were fixed in 10 % neutral-buffered formalin, routinely processed, and embedded in paraffin. The 3-µm-thick sections were mounted on glass slides and stained with haematoxylin and eosin (HE) for light microscopy.

Organ weights and sizes

Weight of body, left lung, spleen, liver, kidney, gonads, brain, adrenal, heart and width ratio of left cardiac ventricular wall and right cardiac ventricular wall, and weight ratio of left and right lung were compared to body length (straight line from tip of snout to fluke notch). For each comparison, the best fit line with the highest R^2 (coefficient of determination) was plotted by use of Excel (Microsoft office; linear, exponential, polynomial, logarithmic, or power). To investigate if extreme variation from the mean contained relevant information, the 5 % most extreme values (high or low) were checked for diagnoses and probable causes of stranding for each organ or ratio. Whether a value was extreme was determined by the difference between measured and predicted or absolute value. For relationships with an $R^2 > 0.50$ (indicating body length was an independent with a strong predictive value for the variable measured) predicted R^2 values were chosen. Absolute measured values were chosen in case $R^2 < 0.50$ (indicating body length had weak predictive value for organ weight).

Bacteriology

For bacteriological examination of animals displaying gross or histological lesions suggestive of bacterial disease, samples of lung, kidney, liver, spleen, pulmonary lymph node, and adrenal gland were frozen at –20 °C and after thawing, cultured according to a standard protocol. Briefly, each tissue was plated on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK), MacConkey agar (Oxoid), and Farrell's medium [153], which was set up specifically for the recovery of *Brucella ceti* [154]. A chocolate agar (CA) plate (Oxoid) was included for lung and pulmonary lymph node. CSBA, CA and Farrell's plates were incubated at 37 °C aerobically plus 5 % CO_2 and examined daily for 14 days, whereas MacConkey agar plates were incubated aerobically without added CO_2 at 37 °C for 48 hours. Isolates were identified based on Gram stain reaction and morphology, gaseous requirements and a range of phenotypic tests, dependent upon the suspected identity of each isolate. Phenotypic tests included classical methods and commercial API identification kits (BioMerieux, Basingstoke, UK), which included analytical profiles for bacterial species from marine mammals established in-house.

Parasitology

Parasites were sampled and preserved in 70 % ethanol. Parasite abundance per porpoise per organ (or organs in case of left and right lungs) was estimated and either classified in two categories (light, 1-100 parasites; heavy, > 100 parasites) infection or four categories (1-10 parasites; 11-100 parasites; 101-1000 parasites; > 1000 parasites). Nematode length and width were measured. Pulmonary nematodes were specified according to length and host organ infected based on previous research by Gibson and others [32]: nematodes < 30 mm, *Stenurus minor*; 30 – 70 mm, mixed *Torynurus convolutus* and *Halocercus sp*; > 100 mm, *Pseudalius inflexus*.

To assess the role of parasitic infections in the lungs as a cause of pneumonia, the presence and abundance of parasites in the lungs were compared between animals with and without pneumonia as a probable cause for stranding, per parasite species and per age category, using the Fisher test (two-sided). A $p < 0.05$ was considered as a significant difference in prevalence and intensity of infection.

Virology

As morbillivirus infections have been identified as a cause of deaths among harbor porpoises [72] lung and spleen samples of all animals were tested by reverse transcriptase polymerase chain reaction (RT-PCR) for the presence of morbilliviral RNA. Total nucleic acids were isolated from 300 µl of a 10 % organ homogenate using the High Pure Viral Nucleic Acid Kit (Roche diagnostic GmbH, Mannheim, Germany), following the protocol provided by the manufacturer. After first strand synthesis, morbillivirus-specific primers P1: 5'ATGTTTATGATCACAGCGGT3' and P2: 5'ATTGGGTTGCACCACTTGTC3' were used for PCR. PCR reactions were checked on 2 % agarose gels.

Grey seal attack bite marks

Photographs of suspect lesions of the integument were evaluated according to criteria set by Leopold et al. [138] by one of the co-authors of that article (Begeman).

Selection of significant lesions and diagnoses

Significant lesions were those lesions considered responsible for stranding by themselves or together with other significant lesions in the same animal. Selection of significant lesions was based on combined analysis of clinical observations and pathological results. A significant diagnosis was defined as a diagnosis based upon the observation of a significant lesion. Significant diagnoses or lesions acquired whilst in rehabilitation, for example an aspiration pneumonia due to tube feeding, were ignored in this manuscript. Incidental diagnoses were those diagnoses based upon the observation of lesions, which were considered too minor to have contributed to stranding. These diagnoses are not further discussed here, but are available in the supplementary material (Additional file 1 table 1).

RESULTS

Harbor porpoises rescued and autopsied

The total number of animals admitted for rehabilitation between 2003 and 2016 was 131, of which 61 (47 %) were autopsied following death or euthanasia. Forty-three animals were autopsied fresh after having been put on ice immediately after death. They were autopsied within 24 hours of death (36 animals) or between 24 and 72 hours after death (7 animals). The remaining 18 animals were frozen immediately after death (-20 °Celsius) and autopsied at a later date. Animals autopsied originated from the North Sea coasts of Germany (7), Belgium (2) and the Netherlands (52). Juveniles were the main category of both admitted (106/131; 81 %) and autopsied (43/61; 70 %) animals, while males and females were more or less equally represented, except in neonates, where only males were presented for rehabilitation (Table 1). Numbers of animals varied between 3 to 10 admitted and 2 to 5 autopsied annually, except for 2006, 2011, and 2012, which had exceptionally high numbers of admissions (18, 15 and 15 respectively) and parallel high numbers of autopsies (12, 6 and 7 respectively). There were more admissions in winter (n = 66) than in spring, summer or autumn (n = 26, 17, 22 respectively; additional file 2).

Table 1 Sex and age category of admitted and autopsied harbor porpoises between 2003-2016

Age class	No. admitted (no. M/no. F)	No. autopsied (no. M/no. F)
Neonate	5 (5/0)	4 (4/0)
Juvenile	106 (53/53)	43 (21/22)
Adult	20 (8/12)	14 (4/10)
Total	131 (66/65)	61 (30/31)

Overview of significant diagnoses

Pneumonia was the only significant diagnosis in 13/61 (21 %) animals and one of multiple significant diagnoses in 21/61 (34 %) animals (table 2 and additional file 1 table 2). The cause of pneumonia was determined in 30/34 (88 %) animals: 13 due to parasitic infection combined with bacterial or fungal infection; 8 due to parasitic infection alone; 6 due to bacterial infection alone, 4 due to fungal infection alone, and 1 due to aspiration of gastric content. In most animals with pneumonia as a significant diagnosis, gross lesions were evident (Figure 1). The typical character and distribution of the gross lesions differed among pneumonias of parasitic, bacterial, and fungal aetiology, although there was some overlap. Histologically, the differences were more distinct (Figure 2, description in additional file 1).

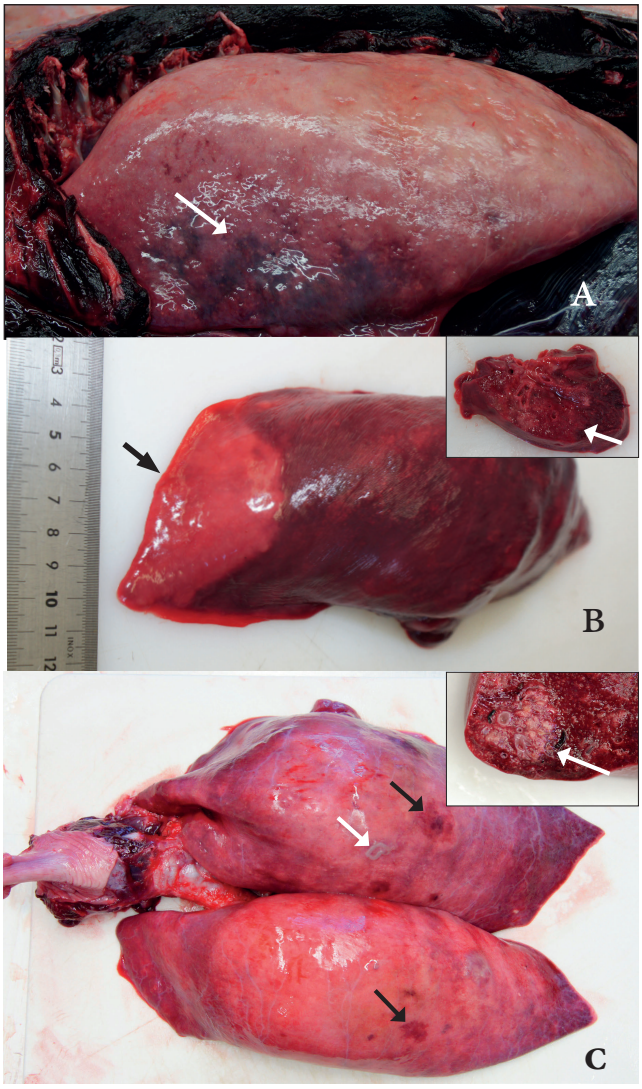

Figure 1 Macroscopic aspects of pneumonias of varied aetiology in harbor porpoises

A bacterial pneumonia, a focal purple colored lesion is present in the ventro-cranial part of the left lung. The white arrow points to the lesion. **B** Fungal pneumonia, a yellow sharply demarcated lesion is present at the caudal tip of the right lung. Insert shows lesion visible at cut surface. Arrows point to lesions. **C** Parasitic pneumonia, multiple nodules of less than 1cm diameter, some associated with a hyperaemic region surrounding or adjacent to the nodule are visible at the surface. Black arrows point to nodules, white arrow points to subpleural a scar caused by a calcified nematode. Insert shows lesion at cut surface, which is a poorly demarcated firm yellow nodule. Extensive gross and histologic description available in supplementary material.

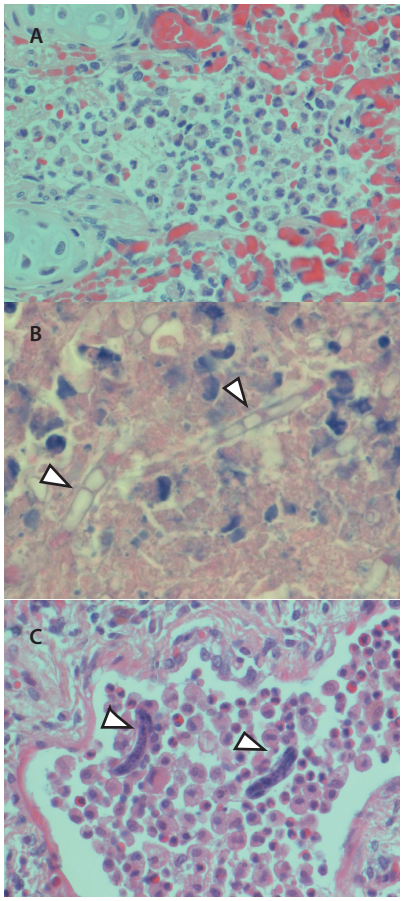

Figure 2 Distinct histopathological features of pneumonia from different causes.

A: Bacterial pneumonia in porpoise PP140917. Neutrophils and fibrin fill an alveolar lumen. **B**: Fungal pneumonia in porpoise PP121130. Fungal hyphae (arrowheads) with internal segments are present in cellular debris at the edge of a pulmonary abscess. **C**: Parasitic pneumonia in porpoise PP040324. Nematode larvae (probably *Stenurus minor)* (arrowheads), macrophages, and eosinophils fill an alveolar lumen. Haematoxylin and eosin. Original magnifications: 40 X objective (A, C); 100 X objective (B).

Five of 61 (8 %) animals had only one significant diagnosis based upon a single organ other than the lung (Table 2). Diagnoses were: pancreatic duct hyperplasia, bite wounds by predators in the integument and bones, ulcerative oesophagitis, hepatic necrosis and lipidosis, and protein-losing nephropathy (Supplementary Online Material).

Twenty-nine of 61 animals (48 %) had significant diagnoses in multiple organs (Table 2 and additional file 1 table 2). Besides the lungs (pneumonia) (n = 21; see above), the main organs affected in animals with multi-organ disease (n = 7 per affected organ) were liver (hepatitis or hepatic lipidosis), brain (encephalitis or encephalomyelitis), and integument (dermatitis or bite wounds). In 7/29 (24 %) of these animals with significant diagnoses in multiple organs, a single aetiology was identified as the cause of the multi-organ disease: fungal infection in 3 animals, with spread from lung or middle ear to brain or pharynx; bacterial infection in 1 animal, with sepsis affecting lungs, muscles and connective tissue; parasitic infection in 1 animal, affecting both lungs and pulmonary blood vessels; bite wounds in 1 animal, affecting both integument and skeleton; and a metabolic disorder in 1 animal, affecting both liver and kidney.

Table 2 Organs affected, morphological diagnosis, and aetiology of significant diagnoses observed in 61 harbor porpoises

		No. of animals with severe lesion in specified organ										
		Inflammatory lesion from:										
Organ	**Total (single / multiple)**	**Nematodes**	**Nematodes plus bacteria**	**Nematodes plus fungi**	**Bacteria**	**Bacteria plus fungi**	**Fungi**	**Viruses**	**Viruses plus bacteria**	**Unknown micro-organisms**	**Unknown cause**	**Non-inflammatory lesion**
Lung	35 (13/22)	9	7	3	5		4[b]			1	5	1
Liver	7 (1/6)				1						4	2
Brain	7 (0/7)					1	2[c]	1			3	
Integument	7 (1/6)				4				1		1	1
Kidney	4 (1/3)											4
Ear	3 (0/3)					2					1	
Muscle	3 (0/3)				1							2
Heart	2 (0/2)						1[d]				1	
Pancreas	2 (1/1)										1	1
Skeleton	2 (0/2)				2							
Oesophagus	1 (1/0)											
Eye	1 (0/1)										1	
Pharynx	1 (0/1)						1[d]					
Stomach	1 (0/1)	1										
Vasculature	1 (0/1)											

[a]Single, number of animals with severe lesion diagnosed only in specified organ. Multiple, number of animals with severe lesion diagnosed also in one or more other organs.
[b]In three of these four animals, fungal infection spread from the lungs to other organs.
[c]In one of these two animals, fungal infection spread from the lungs.
[d]In this animal, fungal infection spread from the lungs.

Specific aetiology of infectious diseases

Fungal infections

In all nine animals with significant diagnoses from fungal infections, the aetiology was *Aspergillus* sp. In 6/9 (66 %) animals, the fungus was identified as *Aspergillus fumigatus* by culture, while in 3/9 (33 %) animals, culture was negative and the fungus was identified as *Aspergillus* sp. by histology, based on characteristic morphology. In 7/9 (78 %) animals, the lungs were infected; in 3 animals, aspergillosis was also diagnosed in an additional organ: heart, brain, or pharynx (Table 2). In 2/9 (22 %) animals, aspergillosis was diagnosed in the middle ear and had spread to the brain (Figure 3).

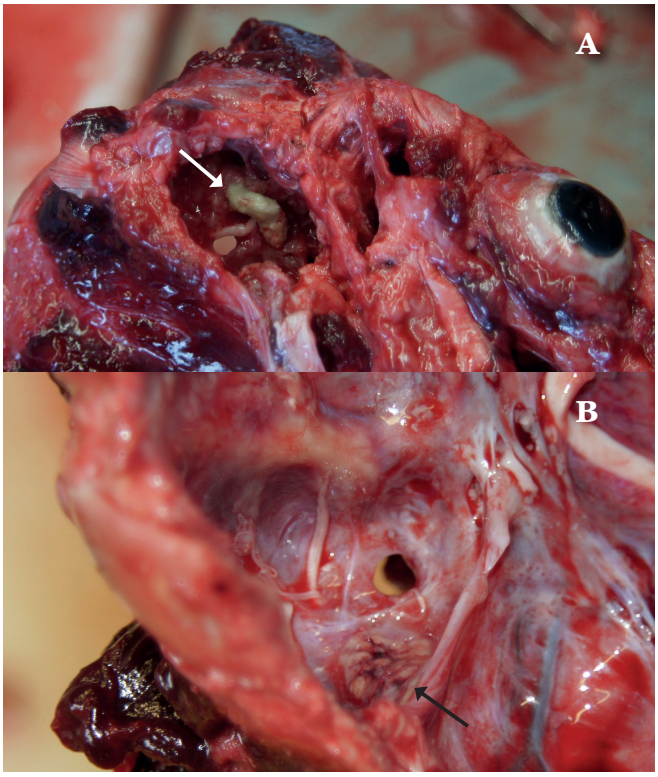

Figure 3 Fungal infection of the middle ear which extends into the cranial cavity.
A View upon the ventral aspect of the skull with the bulla tympanica removed. Green pasty substance is visible (white arrow). **B** View into the cranial cavity adjacent to the inner ear. The inflammation can be seen to extend from the inner ear to the meninges (black arrow).

Viral infections

Two significant diagnoses of viral aetiology were made, one in the brain and the other in the integument. Lung and spleen samples of all animals (n = 61) tested negative for the presence of morbilliviral RNA by RT-PCR.

The case with a viral infection of the brain suffered from lymphocytic encephalitis with neuronal necrosis and intranuclear inclusion bodies. It was diagnosed as *Phocoena phocoena* herpesvirus type 2, based on a combination of PCR, virus culture, histology, and electron microscopy [155].

The case with a viral infection of the integument suffered from multifocal pyogranulomatous dermatitis (Figure 4). It was suspected to have been caused by a papillomavirus infection based on characteristic histological changes, including epidermal hyperplasia, keratin pearls, invagination of the epidermis and associated increase in vascularization of the subjacent dermis. Bacterial infection and associated inflammation were also present, and were considered to be secondary to the viral infection.

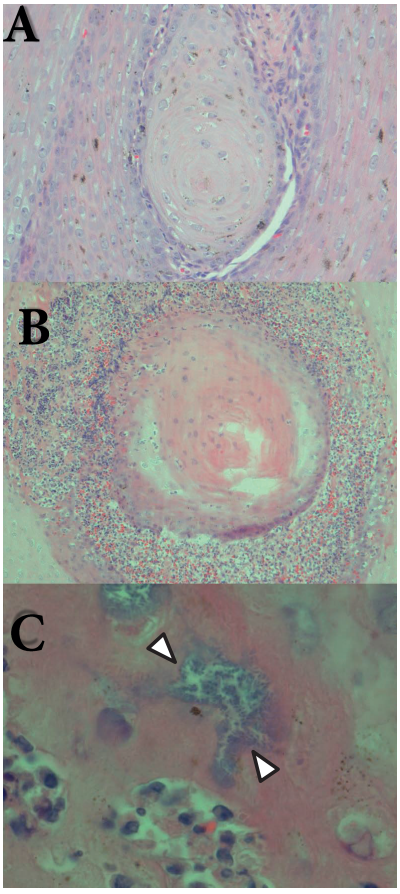

Figure 4 Histopathological features of the pyogranulomatous dermatitis in porpoise PP121130.

A: Keratin pearl, consisting of concentric rings of squamous cells with progressive keratinization towards the centre, within the epidermal layer. **B**: There is infiltration of many neutrophils and macrophages at the border between keratin pearl and surrounding epidermis. **C**: There is an aggregate of bacteria (in between arrowheads) among the infiltrating inflammatory cells . Haematoxylin and eosin. Original magnifications: 20 X objective (A); 10 X objective (B); 100 X objective (C).

Bacterial infections

In 12 animals, bacterial infections lead to significant diagnoses (Table 3). These bacterial infections were considered to be the cause of the observed lesions because infiltration of many neutrophils, with or without macrophages and syncytia, were seen on histology with or without bacteria visible, and bacteria were cultured from samples of the lesions. The diagnoses associated with these bacterial infections were pneumonia in nine animals, sepsis in two animals, dermatitis in two animals, and otitis combined with panencephalitis in one animal. A single bacterial species was responsible for infection in eight animals, while multiple bacterial species were responsible in four animals. *Streptococcus sp.* and *Enterococcus faecalis* were each isolated in three animals, *Escherichia coli, Actinobacillus delphinicola, Shewanella putrefaciens,* and *Brucella sp.* were each isolated in two animals, and *Salmonella sp., Pseudomonas aeruginosa,* and *Clostridium perfringens* were each isolated in only one animal. The *Salmonella sp.* was a monophasic group B *Salmonella* thought to be adapted to, and specific for, the harbor porpoise [156, 157]

Table 3 Significant diagnoses from bacterial infections in respiratory, lymphoid, central nervous, and integumentary systems.

Erasmus code number	Bacterium	Lesion	Observation which links bacterium to lesion
PP041215	*Aeromonas sp.*	Pneumonia, necrotizing, suppurative, locally extensive, acute marked.	Aggregates of bacteria with neutrophils. *Aeromonas* sp. cultured from lung
PP110329	*Actinobacillus delphinicola*	Bronchopneumonia, multifocal, acute, marked.	Alveoli filled with neutrophils. Part of these neutrophils are degenerate and apparently transformed into globules of dark blue chromatin (as seen with some bacterial infections: nuclear streaming). *Actinobacillus delphinicola* cultured from lung and lung draining lymph node.
PP070221	*Actinobacillus delphinicola Brucella sp.*	Bronchopneumonia, multifocal, suppurative, acute, moderate.	Bacteria observed with fibrin, macrophages, and neutrophils in lung lesions. *Actinobacillus delphinicola* cultured from lung draining lymph node, *Brucella sp.* cultured form lung.
PP110711	*Brucella ceti*	1. Pneumonia, pyogranulomatous, locally extensive, chronic, marked	Marked inflammatory reaction with many neutrophils and macrophages. TBLN sample yielded culture of *Brucella ceti*

Erasmus code number	Bacterium	Lesion	Observation which links bacterium to lesion
		2. Lymphadenitis multifocal subacute to chronic marked.	Subcapsular infiltration with neutrophils (TBLN), increase of lymphocytes in medulla and vacuolated macrophages in cortex (PSLN). *Brucella ceti* cultured from both lymph nodes
PP120906.3	*Enterococcus faecalis*	Interstitial pneumonia, suppurative, histiocytic, locally extensive, chronic, moderate	Inflammatory reaction typical for bacterial infection. *Enterococcus faecalis* cultured form lung sample.
PP030405	*Escherichia coli; Pseudomonas aeruginosa; Streptococcus sp.*	Pneumonia, pyogranulomatous, multifocal, chronic, marked.	Histologic association of bacteria with lesion. *Escherichia coli; Pseudomonas aeruginosa; Streptococcus sp.* cultured from lung sample.
PP061122.2	*Escherichia coli*	1. Bronchopneumonia, suppurative, multifocal, subacute to chronic, marked. 2. Myositis, suppurative, multifocal, acute, moderate 3. Fasciitis, suppurative, focal, acute, moderate	Histologic association of bacteria with lesion. *Escherichia coli* cultured from sample.
PP121031	*Salmonella sp.* (host adapted group B *Salmonella*)	Bronchopneumonia, pyogranulomatous, necrotizing, multifocal, chronic, moderate.	Histologic association of bacteria with lesion. *Salmonella sp.* cultured from sample.
PP120906.2	*Streptococcus dysgalactiae*	1. Interstitial pneumonia, suppurative, diffuse, acute, moderate. 2. Hepatic abscesses, multiple, marked 3. Arthritis, suppurative, diffuse, acute, marked 4. Sepsis	Inflammatory reaction typical of bacterial infection (suppurative interstitial pneumonia, multiple marked hepatic abscesses, observation of strings of bacteria in joint capsule with associated inflammatory relation) *Streptococus dysgalactiae* cultured from ear abscess, liver abscess, lung, liver, spleen, kidney, trachea-bronchial lymph node, pre-scapular lymph node, adrenal and uterus.

Erasmus code number	**Bacterium**	**Lesion**	**Observation which links bacterium to lesion**
PP121130	*Shewanella putrefaciens* *Enterococcus faecalis* Gram negative non-fermenter	Dermatitis, pyogranulomatous, multifocal, chronic, marked, with epidermal hyperplasia, keratin pearls, and bacterial infection.	Many aggregates of small coccobacilli mixed with many neutrophils and macrophages observed in the epidermis.
PP111219	*Escherichia coli* *Stenotrophomonas maltophilia*	Bronchopneumonia, haemorrhagic, suppurative, diffuse, acute, marked, associated with bacterial infection	Histologic association of bacteria with lesion. *Salmonella sp.* cultured from sample.
PP050502	*Shewanella putrefaciens* *Enterococcus faecalis* *Streptococcus sp.*	Dermatitis, multifocal, suppurative, superficial, acute, moderate,	Histologic association of bacteria with lesion and bacteria cultured from sample.
PP060524	*Clostridium perfringens*	1. Cerebellum: panencephalitis, pyogranulomatous, haemorrhagic, necrotizing, locally extensive, marked associated with fungal hyphae (*Aspergillus sp.*) and mixed bacterial infection. 2. Otitis media purulent subacute to chronic diffuse marked.	Bacteria observed centrally in pyogranulomas in meninges and cultured from middle ear and brain samples.

Parasitic infections

Respiratory tract

The pulmonary nematodes *S. minor, T. convolutus, Halocercus sp.,* and *P. inflexus* were found in juveniles both with and without a significant diagnosis of pneumonia (Table 4 and additional file 1 table 3). Prevalences and intensities of both *T. convulutus/Halocercus sp.* infection and *P. inflexus* infection were significantly higher in juveniles with significant diagnoses of pneumonia than in juveniles without severe pneumonia ($p < 0.05$, Fisher test two sided) , but prevalence and intensity of *S. minor* did not differ significantly between the two groups ($p > 0.05$, Fisher test two sided). No significant differences in prevalence and intensity of any of the pulmonary nematode species were found between adults with and without severe pneumonia (Additional file 1 table 4 and 5).

Pulmonary nematodes were not detected in neonates. The estimated age of the youngest juveniles in which pulmonary nematodes were detected was 9 mo. (*S. minor*), 6.5 mo. (*T. convolutus/Halocercus sp.*), and 5 mo. (*P. inflexus*). *Pseudalius inflexus* had a significantly higher prevalence and intensity in adults than in juveniles (above 6 mo. of age) ($p < 0.05$, Fisher test two sided); Infections with *T. convolutus/Halocercus sp.* and *S. minor* did not differ significantly in prevalence and intensity of infection between juveniles (13/42, 31 % infected) and adults (10/12, 83 % infected).

Table 4 Presence and burden of nematode infections in juvenile harbor porpoises with and without pneumonias of different aetiologies

Lung pathology and aetiology	No. of harbor porpoises infected (no. with light infection/no. with heavy infection)		
	Stenurus minor	***Torynurus convolutus / Halocercus sp.***	***Pseudalius inflexus***
No severe pneumonia (n=20)	3 (3/0)	5 (4/1)	1 (1/0)
Severe pneumonia (n=21)	2 (2/0)	14 (5/9)	11 (5/6)
Parasitic (n=6)*	1	4 (2/2)	4 (1/3)
Bacterial (n=5)	1 (1/0)	2 (2/0)	2 (2/0)
Fungal (n=2)**	0	1 (1/0)	1 (1/0)

Light = 1-100 nematodes (both lungs
Heavy = >100 nematodes (both lungs)

Pulmonary vasculature

The prevalence and intensity of *P. inflexus* infection in pulmonary blood vessels was significantly higher in adults (10/14, 71 % infected) than in juveniles (19/41, 46 % infected) ($p < 0.05$, Fisher test two sided; additional file 1 table 6). Histologic lesions of the pulmonary vasculature were observed only in animals with an associated *P. inflexus* infection (Additional file 1). No gross lesions of the pulmonary vasculature were observed. The youngest animal with a *P. inflexus* infection of the pulmonary vasculature was estimated to be 5 mo. of age.

Digestive tract

Parasitic infections in the organs of the digestive tract were not associated with significant diagnoses. *Campula oblonga* infection of the liver and *Anisakis simplex* infection of the forestomach occurred significantly more often in adults than in juveniles ($p < 0.05$, Fisher test two sided). The prevalence of *C. oblonga* infection of the pancreas, *Pholeter gastrophilus* infection of the pyloric stomach and *Diphyllobothrium stemmacephalum* of the intestine did not differ significantly between juveniles and adults ($p > 0.05$, Fisher test two sided). Prevalences of parasitic infections of organs of the digestive tract are

available in additional file 1 (Table 7 and 8). The estimated age of the youngest animals with parasitic infections of the digestive tract were: 9 mo. for *C. oblonga* infection of the liver, adult (of unknown age) for *C. oblonga* infection of the pancreas, 7 mo. for *A. simplex* infection of the fore stomach, 9 mo. for *P. gastrophilus* infection of the fundic stomach and 11 mo. for *D. stemmacephalum* infection of the intestine.

Aetiology of non-infectious diseases

Separation from mother animal

The probable cause of stranding in 10/61 (16 %) animals was separation from the mother in mother-dependent animals. Three of these were neonates, and seven were emaciated juveniles of less than 10 mo. of age, at which time they were still mother-dependent [158]. The diagnosis was based on severe emaciation (only observed in juveniles, not in neonates), together with absence of other lesions that could explain stranding. Emaciation was characterized by atrophy of the epaxial and cervical muscles, absence of internal fat (e.g. around heart and lungs), and a thin blubber layer (less than 15 mm average measured at the circumference cranial to the dorsal fin). Emaciation was externally visible as the dorsolateral surface of the body at the level of the dorsal fin being concave rather than convex, and the presence of a dorsal indentation between head and thorax, rather than a flush transition. In six out of seven emaciated animals, the blubber layer was thinner than 15 mm. Normal blubber thickness is 18 to 20 mm on the thorax [159].

Physical trauma from putative grey seal attacks

In three juveniles and one adult animal, lesions attributable to grey seal attack were observed. These animals stranded in 2011, 2015 and 2016. Lesions occurred on the tail stocks of all animals, on the pectoral flipper of one animal, and on the head of another. The head lesion was so severe that the animal had to be euthanized (Figure 5). Trauma from grey seals was considered to be a significant diagnosis in two animals and an incidental diagnosis in the remaining two.

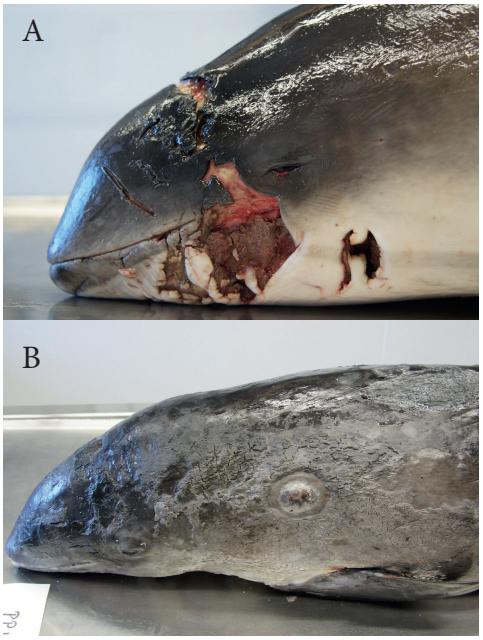

Figure 5 Lesions of the integument.

A Traumatic lesions caused by a grey seal attack on a live(!) stranded harbor porpoise. **B** Generalized inflammatory lesions of the integument caused by a mixed viral and (secondary) bacterial infection.

Organ weights and sizes

We found the following associations between the 5 % highest or lowest organ weights and significant diagnoses: high brain weight with encephalitis; high lung weight with pulmonary congestion; low liver weight with cholangitis; and high adrenal weight with hyper- or hypoplasia of the adrenal gland (Additional file 3).

Comparison of clinical and pathological observations

Clinical observations were made for 48/61 animals during rescue and rehabilitation (Additional file 1 table 9). In some cases, significant diagnoses after death correlated well with clinical signs before death. Animals with a significant diagnosis of only pneumonia (n=7) showed the following respiratory signs: bradypnea (2/7), tachypnoea (2/7), exaggerated breathing movements (1/7) or rhonchi in the bronchi during auscultation (1/7). Animals with significant diagnoses of fungal otitis media and encephalitis (n=2) showed both nervous signs and non-specific clinical signs: uncoordinated swimming behaviour (2/2), vertical nystagmus and delayed pupillary reflex unilaterally (1/2), increased cardiac rate (1/2) and electrolyte imbalance with decreasing total protein in the serum despite good food intake (1/2). Animals with significant diagnoses of both encephalitis and a pneumonia (n=5) showed both respiratory and nervous signs: dyspnoea with forced laboured breathing (3/5), tachypnoea (2/5) and lifting of the entire head out of the water for inspiration (2/5).

In few animals, there were notable discrepancies between significant pathological diagnoses and clinical signs: three animals with nervous signs had no or only mild brain lesions at autopsy, and two animals with clinical signs of kidney failure had only mild kidney lesions at autopsy (Table 5). For most animals with significant diagnoses in multiple organs (n=23), as well as animals with no significant diagnosis (n=4), it was not possible to compare significant pathological diagnoses with clinical signs.

Table 5 Animals with discrepancies between clinical signs and pathological observations

Animal	Clinical signs	Pathology observations
1	Multiple epileptic seizures with loss of control.	No lesions observed
2	Kidney failure, marked increase in urea, creatinine and sodium values with loss of appetite and vomiting.	Nephritis, suppurative, focal, acute, mild Renal medullary calcification, multifocal, mild
3	CNS: Body tremor, forceful difficult expiration Digestive or CNS: Cramps gastric stasis Respiratory or CNS: increased breathing frequency	CNS nad*. Cornea and brain herpesvirus PCR positive Digestive: nad Respiratory: pulmonary oedema (acute agony related)
4	CNS symptoms: hypothermia, disorientated swimming against the wall, laboured breathing with vertical rises above the water to inspire	Cerebrum: polioencephalitis, multifocal, mild.
5	Kidney failure: marked increase urea, creatinine, sodium, vomiting	Kidney: urolithiasis, mild, some protein granules in the collecting ducts

*nad = no abnormalities detected

DISCUSSION

In the present paper we have investigated diseases, of live stranded harbor porpoise, that were severe enough to have contributed to stranding. In comparison with previous surveys [22-24, 26], we observed a higher prevalence of fungal diseases, a higher prevalence of significant lesions in integument, brain, kidney and liver, and a higher prevalence of animals which had significant lesions in multiple organs (Table 6).

Table 6 Comparison of frequency, location and aetiology of causes of death or stranding in harbor porpoises from the North Sea of five autopsy overviews.

Publication	Location of lesion, or reason responsible for stranding or death								
	Lungs	Starvation	Brain	Liver	Integument	Kidney	Sepsis	Unknown	Other
British waters 1979-1991*[26] (n=31) 21 neonates, 30 juveniles, 49 adults									
Total prevalence	**45**	**10**				**3**	**6**	**10**	**32**
Parasites	23								
Bacteria	13						6		
Fungi	3								
Viruses									
Non inflammatory	6					3			
German North and Baltic seas 1991-1996#,*[23] (n=66) 5 foetuses, 35<0.5years, 0.5<41<4years, 4years<20									
Total prevalence	**46**	**<7**	**?**	**?**	**?**	**?**	**?**	**24**	**?**
Parasites plus bacteria	Mainly								
Fungi	2								
Belgium and Northern France 1990-2000*[22] 11 neonates, 57 juveniles, 32 adults									
Total prevalence	**56**	**9**	**4**				**2**	**?**	
Parasites	26								
Bacteria			2				2		
Parasites plus bacteria	30								
Unknown			2						

England and Wales 1990-1995*[24] (n=104) ages not specified									
Total prevalence	**28**	**20**	**2**	**1**			**7**	**23**	**19**
Parasites	8								
Bacteria	6						6		
Parasites plus bacteria	8								
Fungi	2								
Viruses							1		
Non inflammatory									
Unknown	5		2	1					
Dutch and adjacent coasts 2003-2016 (n=61) 7 neonates, 70 juveniles, 23 adults									
Total prevalence	**58**	**16**	**11**	**11**	**13**	**7**	**5**	**7**	**20**
Parasites	15								
Bacteria	10			2	7		5		
Parasites plus bacteria	11								
Fungi	7		3						
Fungi plus parasites	5								
Fungi plus bacteria	11		2						
Viruses			2						
Viruses plus bacteria					2				
Non inflammatory	2			3	5 (trauma)	7			
Unknown	8		5	7					

Between brackets are number of animals autopsied. All other numbers are percentages. Bold underscored numbers are total percentages.
Prevalence for causes of stranding for the publication referring to the Dutch coast, prevalence for cause of death for the four other publications.
***** Bycaught animals, decomposed animals and animals dead due to suspected bycatch related trauma excluded
# Results reported for mixed bycaught and stranded animals, only results reported for which it was clear they related to stranded animals
?= could not be deduced from publication

Our study noted a high prevalence of fungal infections, which were mostly caused by *A. fumigatus*, and used the lungs or middle ears as portal of entry. The prevalence of fungal diseases was higher in our study (15 %) than in previous surveys (2 to 3 %) [22-24, 26] and in a study of pulmonary pathology of stranded harbor porpoises (5 %) [33]. All 9 infections noted by us were caused by *Aspergillus* sp. derived from the typical histologic appearance of the fungal hyphae. In six cases we could further identify the species to *Aspergillus fumigatus* by successful culture. Comparison to previous research is difficult as successful cultures are only reported sparsely. Siebert et al noted one mycotic infection caused by *Rhizopus sp.* and Jepson et al. were able to culture *Aspergillus terreus* in one case. *Aspergillus fumigatus* has been identified in a middle ear infection and a brain infection [160, 161] and *Aspergillus terreus* has been identified in a middle ear infection [162]. Most infections we observed (7/9) were fungal pneumonias which spread via the blood to brain, heart and pharynx in three separate cases, analogous to the dissemination of invasive aspergillosis in humans [163]. The two other cases we observed were fungal middle ear infections, which spread per continuitatum to the brain. In these two cases consequences were clearly visible in the live animals which showed neurological signs such as erratic swimming behaviour, vertical nystagmus and unnatural body posture. Our and previously reported middle ear infections in harbor porpoises [161, 162] were infections without evidence of pulmonary involvement. We speculate that infectious *Aspergillus* conidia in inhaled air entered the middle ear via the Eustachian tube. Compared to terrestrial mammals, Eustachian tubes in cetaceans are broader and firmer. This anatomical adaptation is thought to guarantee airflow and thus prevent barotrauma when diving [164], but also might act as an efficient portal of entry for *Aspergillus* infections.

We speculate that the higher prevalence of aspergillosis we observed in harbor porpoises is caused by impaired immunity rather than increased exposure to infectious *Aspergillus* conidia or more sensitive diagnosis of aspergillosis. Impaired immunity due to host damage is a requirement for the development of invasive aspergillosis. In the immunocompetent host, defence mechanisms show a striking redundancy [165]. Although prevalence of *Aspergillus* infections is related to infection pressure in birds [166], there is no reason to assume that the higher prevalence of aspergillosis we observed in harbor porpoises is due to increased infection pressure from *Aspergillus* conidia for harbor porpoises in recent decades. An increased amount of decaying plant material and composting facilities around coastal areas would provide justification for such a suspicion. To the best of our knowledge these changes have not occurred in countries around the North Sea. It is also unlikely that the higher prevalence of aspergillosis is due to more sensitive diagnosis of aspergillosis. Gross lesions were clearly visible and histologic confirmation was straightforward (Figures 2 and 3).

Impaired immunity, responsible for the increased prevalence of aspergillosis, could be caused by anthropogenic pollutants, viral infections or malnutrition. The influence of anthropogenic pollutants on the immune system of harbor porpoises is a possible cause

for impaired immunity. Several investigations have found a positive correlation between high levels of heavy metals and PCBs in harbor porpoise tissues and the prevalence of infectious diseases [144, 145]. Hall et al. quantified the increase in risk of mortality due to infectious disease by the concentration of PCB congeners in the blubber layer of harbor porpoises. They stated that a concentration above 25 mg/kg lipid put the animal at an increased risk of mortality [167]. Individuals from our study were well above this 25 mg/kg threshold as was observed by Weijs et al. [146]. Dutch coastal waters are the first to receive the heavily polluted waters from the rivers Rhine, Meuse, Waal, and Eems and have higher levels of PCBs than other regional areas [168]. Another possible cause for impaired immunity is virus induced immunosuppression. We did not observe any infections with morbillivirus, which has been documented as a likely immunosuppressant virus in harbor porpoises. We may have overlooked infections with other viruses. Viral infections can easily be overlooked as knowledge about which viral infections occur in marine mammals is far from complete and one therefore does not know what to look for nor does one know where to look. Finally, malnutrition is a well-known cause for impaired immunity[169]. Our data set did not allow the assessment of sub-lethal effects, such as impaired immunity, by malnutrition.

We observed that a parasitic infection was not a prerequisite for a bacterial pneumonia, as speculated previously [22, 23]. Pure bacterial pneumonias occurred independently of prevalence or intensity of parasitic infections. Harbor porpoises with bacterial pneumonias and without pneumonias had the same prevalence and intensity of pulmonary parasitic infections with *S. minor, P. inflexus* or *Halocercus sp.* and *T. convolutus.* (Fisher test > 0.05; additional file 1 table 5). However, we did find that an increased prevalence and intensity of parasitic infections of the lung with *P. inflexus* and *T. convolutus* and *Halocercus sp.* were associated with fungal and combined bacterial-parasitical pneumonias (Additional file 1 table 5).

Infestation of airways with a large number of parasites did not necessarily cause clinical or pathological evidence of disease. Some researchers speculated that pulmonary parasites blocked bronchi and thereby caused fatal respiratory problems [23, 26]. However, we observed harbor porpoises with heavy infestation with *P. inflexus* or *Halocercus sp.* and *T. convolutus* (Additional file 1 table 3 and 4) without any associated clinical signs or lesions. Our observations concur with the observations by Kirkwood et al. [24] and Clausen et al. [28], who noted that harbor porpoises were able to tolerate large numbers of lungworms. We found that prevalence and intensity of *P. inflexus* infection increase with age: these values were significantly higher in adults than in juveniles. Because prevalence and intensity of *P. inflexus* infection are correlated with frequency of pneumonia, this means that results on pneumonia and lung parasitism should be presented separately for each age class: neonates, juveniles and adults. The comparison of our study to previous studies with regard to pulmonary parasitic infections was complicated by the fact that previous surveillance studies categorized infections as mild, moderate or marked, without

quantifying the actual numbers of parasites in each of these categories [22-24, 26]. We feel that such quantification is important to investigate the impact of pulmonary nematodes on pulmonary health.

We observed a higher prevalence of significant lesions in integument, brain and liver than were indicated in previous studies [22-24, 26] (Table 6). In the integument, we observed significant lesions in 13 percent of the animals, mostly due to bacterial infections (5/8 animals); our report stands alone in this. Baker et al. reported lesions of the integument (trauma, non-specific and viral) in 40 percent of the animals in his study but considered these lesions to be non-fatal [26]. Jauniaux et al. reported ulcerative skin lesions in 20 percent of the animals, with severe lesions in 4 percent, but did not clarify whether these lesions were considered severe enough to cause stranding or death [22]. Siebert et al. reported suppurative or necrotizing inflammation of the integument in 8 percent of the animals, but did not clarify whether these lesions were considered significant or incidental, and whether they had occurred in stranded or by-caught animals [23].

In the brain, we observed significant lesions in 11 percent of the animals. Neither Baker et al. [26] nor Siebert et al. [23] reported brain lesions. Kirkwood et al. [24] observed brain lesions in 2 percent of the animals and Jauniaux et al. [22] reported brain lesions in 4 percent of the animals. In previous studies, carcinoma [170], *Toxoplasma* [171, 172], and *A. fumigatus* [160] have been diagnosed as causes of brain lesions. In comparison, we observed *A. fumigatus* or *Aspergillus* sp. infection in half of the brain cases, and no cause in the other half, except for one case of *Phocoena phocoena* herpesvirus type 2 infection [155]. We may have missed some brain lesions by routine histological examination of one location each from cerebrum, cerebellum and brain stem, as nervous signs without supporting pathological diagnoses in the brain were observed in 5 percent of the animals (Table 5). Therefore, more extensive histological examination of the brain is warranted to increase the detection of brain lesions in harbor porpoises.

In the liver, we observed significant lesions in 11 percent of the animals. In previous surveillance studies [22-24, 26], only Kirkwood et al. [24] observed lesions in the liver as a cause of death, and that was in only 1 percent of the animals. Liver lesions in harbor porpoises have been reported by other researchers. Hiemstra et al. [173] noted liver lesions not caused by parasitic infection in 32 stranded harbor porpoises, and Herder et al. [171] described a hepatitis caused by a generalized *Toxoplasma* infection .

A possible explanation for the observed differences in prevalences of significant lesions in liver, brain, kidney, and integument between our study and previous surveillance studies [22-24, 26] may at least in part be due to different approaches to assigning diagnoses. Kirkwood et al. strictly assigned a single cause of death to each single animal [24]. Baker et al. diagnosed 30 lesions as being responsible for death in 28 animals, but did not indicate the diagnoses per animal [26]. Siebert et al. [23] and Jauniaux et al. [22] provided a broad outline of all lesions encountered, but did not indicate clearly which lesions, and in which frequencies, they considered responsible for death. In our study, we assumed that

significant diagnoses in multiple organs may have operated together to cause stranding; we recorded significant diagnoses in multiple organs in 48 percent (29/61) of the animals. Our assumption is in line with that of Wobeser [174], who stated that "while we tend to think about diseases one at a time, wild animals are affected by many different agents, often simultaneously." In our view, Wobeser's statement also holds true for causes of stranding in harbor porpoises.

Starvation was the second most frequent cause for stranding or death in all studies (Table 6; [22-24, 26]). One point to note is that almost all animals, with the exception of two adults, in the study by Jauniaux et al. were neonates or juveniles [22] and the oldest juveniles were around weaning age. This suggests that starvation was mainly due to separation of the juvenile or neonate from the mother and subsequent inability to forage adequately. No adults or independent juveniles were found with signs of starvation, with the exception of the two animals mentioned above. It seems that food shortage does not cause direct starvation in independent harbor porpoises. However, sublethal effects of malnutrition, for example on immunity or fecundity, cannot be assessed by analysis of the data available in this investigation.

Our study on live-stranded harbor porpoises differs from previous surveys, in which dead-stranded harbor porpoises were examined [22-24, 26]. This raises the question of whether it is valid to compare the two. These two samples might differ if a significant number of dead stranded animals died due to diseases which caused death so rapidly, that they would not have had the opportunity to be found stranded alive. A well-known and frequent cause of acute death in harbor porpoises is bycatch [22-24, 26]. Because the vast majority of bycaught animals may be expected to die in the net and strand dead, we excluded known and suspected bycaught animals in the discussion and table 6. Another potential cause of acute death in cetaceans is sepsis [175]. However, the percentage of sepsis encountered in our investigation is relatively small (less than 7 percent) and similar to the percentage encountered in previous surveillance studies on dead stranded animals (Table 6; [22-24, 26]). Therefore, we retained this cause of death in our comparison between live- and dead-stranded animals. A caveat that is valid for all studies on stranded animals, is that the sample of live-stranded harbor porpoises is expected to originate mainly from the part of the population staying close to shore, and it is unknown whether this part of the population is representative for the entire harbor porpoise population.

Recommendations for future research

Concerning diagnostic evaluations among live stranded harbor porpoises, our main recommendations are to conduct:

- more research on *Aspergillus* sp. infections in harbor porpoises;
- more research on the immune system in animals with aspergillosis, focussing on immune organs (lymph nodes and spleen) and cellular immunity (e.g. T lymphocytes)
- more extensive histopathological analyses of the brain and to better quantify pulmonary parasitic infections as part of the autopsy protocol;
- more uniform reporting of diagnoses, in order to facilitate analysis and comparison of different autopsy surveys of harbor porpoises.

The most concerning finding of our study was an apparent increase in *Aspergillus* sp. infections as a cause of stranding in comparison with similar studies in the past. Future research should investigate whether this increase is consistent over time and across different regions of the North Sea, and to determine the causes of increase, including impaired immunity.

The brain deserves proper attention as in many cases it is the organ, which carries lesions responsible for stranding or death. Present autopsy protocols regularly fail to identify lesions, which cause nervous signs in live animals, or fail to identify the aetiology of brain lesions that are identified. Sampling protocols should be reassessed and possibly larger numbers of samples should be taken routinely for histology and tissue banking. Bacterial culture of brain samples should be done routinely. Modern diagnostic techniques like RT-PCR and deep sequencing should be considered for more sensitive diagnosis of known infectious agents, and discovery of novel infectious agents. The biggest potential for success is with the search for viruses as causes of disease, as virus infections can be more difficult to identify during gross necropsy or histology than bacterial, protozoal or fungal infections.

Extensive autopsy programs are useful for conservation of species and the environment. They may help to recognize causes or changes in causes of morbidity and mortality and relate these to (anthropogenic) environmental stressors. The effects of environmental stressors on prevalence of disease agents may be subtle and difficult to note in harbor porpoises, which often have multiple lesions [77]. In order to discern these effects and allow comparison among geographical regions, long-term autopsy programs will be necessary to both provide adequate detail and present their results in a uniform manner [176, 177]. Program reports should make sufficiently clear at which frequencies diagnoses occur. Pneumonias should be reported separately for different age classes and quantification of intensity of pulmonary parasitic infections should be provided. A clear differentiation should be made between significant and incidental diagnoses.

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Availability of Data and Materials:

All data generated or analysed during this study are included in this published article (and its additional files).
Additional files available at: https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-019-0706-3#Sec30

Competing interests

The authors declare that they have no competing interests.

Authors' contributions and consent

CvE participated in the design of the study, carried out the autopsies and drafted the manuscript. MvdB carried out the polymerase chain reaction and assisted in drafting the manuscript. PvR assisted with the autopsies and technical assistance for histology and assisted in drafting the manuscript. PB collected clinical data. JM collected clinical and husbandry data. GF did the bacteriological examinations and assisted in drafting the manuscript. AO provided expertise on virology and assisted in drafting the manuscript. TK conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional file 1

Pathology per organ

1.Respiratory tract pathology:

Pneumonias:

i. Parasitic pneumonia gross lesions and histology,

ii. Bacterial pneumonias gross lesions and histology.

iii. Fungal pneumonias gross lesions and histology.

2. Pathology of the pulmonary vasculature.

3. CNS pathology:

a. Fungal infection, histology,

b. Viral infection, histology,

c. Inflammation of unknown aetiology, histology.

4. Liver pathology:

a. Non inflammatory lesions, gross lesions and histology,

b. Bacterial infection, gross lesions and histology.

Organ sizes and weights (relative to body length) in relation to lesions observed.

Table S1.

Individual animals with their lesions distributed into lesions which contributed to stranding, did not contribute to stranding or were acquired after stranding, with comment interpreting the severity of lesions.

Table S2.

Organs involved and etiological categories involved in significant diagnoses, per stranded harbour porpoise.

Table S3.

Nematode infections in juvenile harbour porpoises with and without severe pneumonia.

Table S4.

Nematode infections in adult harbour porpoises with and without severe pneumonia.

Table S5.

p values according to Fisher's exact test (two-sided) comparing nematode infections in juvenile harbour porpoises with severe pneumonia to those in juveniles without severe pneumonia ($n = 20$).

Table S6.

Comparison of prevalence and abundance of *Pseudalius inflexus* infections in the pulmonary vasculature of juveniles and adult harbour porpoises.

Table S7.

Comparison of prevalence of gastrointestinal parasites in juvenile and adult harbour porpoises.

Table S8.

Comparison of prevalence and abundance of different parasite species in the digestive tracts of juvenile and adult harbour porpoises.

Table S9.

Overview of lesions, diagnosis and most prominent clinical signs.

Additional file 2.

Number of annual admissions according to season, age class and gender.

Additional file 3.

Graphs of organ weights and sizes in relation to body length.

ADDITIONAL FILE 1

PATHOLOGY PER ORGAN

Parasitic pneumonia (n=8):

Gross lesions:

Lung lesions associated with adult nematodes (probably *Halocercus invaginatus*) were characterized by the presence of multiple nodules (range: 3 to 20 per animal). Typically, these nodules were between 5 to 10 mm in diameter, yellow, white, or grey, firm to hard, and poorly demarcated. Those nodules at the lung surface protruded a few mm above the surrounding lung tissue. The lung parenchyma around some nodules was discoloured brown to purple (haemorrhage). On section, some nodules had tightly coiled nematodes, pus, or both in the centre.

Lung lesions associated with larval nematodes (probably *Halocercus invaginatus* or *Stenurus minor*) were characterized by a locally extensive area of consolidated, purple lung parenchyma. On section, pus exuded from bronchiolar lumina. In one adult female a thick-walled abscess surrounding a parasite with spikes on the cuticle and a filled coelomic cavity (suspected trematode) was found, a trematode egg was observed in the lungs of a juvenile female.

Histology

Lung lesions associated with adult nematodes (probably *Halocercus invaginatus*) (n=7) were centred on multiple distinct aggregates of a few adult nematodes coiled up in the alveoli, bronchioles or bronchi. In two animals, these nematodes were surrounded by aggregates of macrophages and neutrophils, with or without multinucleated giant cells. These were diagnosed as chronic, multifocal, pyogranulomatous pneumonia. In two other animals, abscesses had formed, in which the nematodes were surrounded by an inner layer of fibrous connective tissue and an outer layer of macrophages, neutrophils, eosinophils, and lymphocytes. These were diagnosed as pulmonary abscessation.

Lung lesions associated with larval nematodes (probably *Halocercus invaginatus* or *Stenurus minor*) were characterized by diffuse flooding of alveolar and bronchiolar lumina by many neutrophils and few macrophages, mixed with larval nematodes. This was diagnosed as acute, locally extensive, suppurative pneumonia.

Bacterial pneumonias (n=8 with parasitic pneumonia, n=6 without parasitic pneumonia):

Gross lesions:

Lung lesions associated with bacterial infections varied in gross appearance. In 4 animals, no gross lesions suggestive of bacterial infection were observed. Where bacterium-associated lesions were seen, they had three patterns: locally extensive and centred on the cranioventral part of the lungs (n=5), diffuse across both lungs (n=3), or multifocal in

both lungs (n=1). In these animals, lesions were usually purple (other colours were red, grey, or black), heavy, firm, and swollen. On section, the lesional lung tissue was well-demarcated from normal tissue, bulged, and exuded opaque, watery red or viscous yellow fluid. Samples of lesional lung tissue just floated or sank in water. In the one animal with multifocal lesions, some of the lesions had formed abscesses.

Histology
Lung lesions associated with bacterial infections typically were centred on the bronchioles. In the lumina and walls of alveoli, br*onchioles, and bronchi, there were diffuse or multifocal aggregations of many neutrophils and fewer macrophages, often mixed with oedema fluid, fibrin, erythrocytes, and variable numbers of bacteria. In some animals, the central parts of focal lesions were necrotic and there was lysis of neutrophils. The lesions were typically diagnosed as acute, multifocal, suppurative bronchopneumonia.*

Fungal pneumonias (n=7):
Gross lesions:
Lung lesions associated with fungal infections varied in gross appearance. In two animals, no gross lesions suggestive of fungal infection were observed. Where fungus-associated lesions were seen (n=5), they usually consisted of multiple, round or irregular, yellow, well-demarcated, raised, firm nodules, which ranged from 3 to 6 cm in diameter, and sank in formalin. On section, the lesional lung tissue exuded yellow to yellow-green, opaque, viscous fluid (pus), except for one animal, where it was dry and pink (necrosis). In another animal, the lesions were walled off by a 0.5-cm-thick layer of connective tissue (abscesses).

Histology:
Lung lesions associated with fungal infections typically consisted of focal or locally extensive areas of inflammation and necrosis in the lung parenchyma. The lumina of alveoli and bronchioles contained many neutrophils and macrophages, mixed with erythrocytes, fibrin, oedema fluid, and cellular debris. Within the inflamed tissue, there were one or more foci of necrosis of both lung tissue and inflammatory cells. Also, within the lesional tissue, there were multiple parallel-walled, septate fungal hyphae of 5 to 10 microns thick. Some hyphae split into two branches of equal thickness at an angle of less than 90 degrees, and a few hyphae had fruiting bodies (*Aspergillus* sp.). Fungal hyphae were detectable by H&E stain, but much more obvious by Grocott stain. In one animal fungal structures included buds which were reminiscent of a cryptococcus infection. Culture of infected tissue however resulted in the growth of *Aspergillus fumigatus.* In one animal, the largest focal lesion was partially encapsulated by fibrous connective tissue. In another animal, the lesion extended to the pleura, which was diffusely thickened by many macrophages. The lesions were typically diagnosed as chronic, multifocal or coalescing, pyogranulomatous, necrotizing pneumonia.

Pathology of the pulmonary vasculature

In 29 out of the 57 adult and juvenile harbour porpoises a *Pseudalius inflexus* infection of the pulmonary vasculature was observed. Only in animals with a *Pseudalius inflexus* infection were lesions of the pulmonary vasculature observed. In one animal these lesions were considered to be severe (and thus responsible for stranding). No gross lesions of the pulmonary vasculature were observed with the exception of the presence of specimens of *Pseudalius inflexus*. Histologic lesions associated with *Pseudalius inflexus* infection of the pulmonary vasculature were: phlebitis (n=9), semi obstructive infestation (n=1), smooth muscle hyperplasia, multifocal, chronic and marked (n=1) and thrombosis (n=2). Phlebitis was further characterized as diffuse and fibrosing (n=1), suppurative (n=1), proliferative (n=2). Thrombosis was further characterized as suppurative (n=1) and partly to completely obliterative, chronic, active associated with focal fungal infection.

CNS pathology

Fungal infection (n=3)

Histology

Brain lesions associated with fungal infections were not detected grossly, but only by histological examination. In all three cases, the causative fungus had morphological characteristics typical for *Aspergillus* sp. In the more marked lesion, a large part of the tissue section of cerebellum was necrotic and largely replaced by neutrophils and macrophages, with aggregates of lymphocytes around remaining blood vessels and multiple haemorrhages. The overlying pia mater and dura mater was thickened due to fibroplasia and infiltration by lymphocytes, plasma cells, macrophages, and neutrophils. Throughout the affected tissue, and clearly demonstrated by Grocott stain, were fungal hyphae, characterized by parallel walls, septa, and acute branching at about 30 degrees. Additionally, by Gram stain, multiple aggregates of Gram-positive coccoid and rod-shaped bacteria were present. In the less marked lesion, there were multiple aggregates of neutrophils and macrophages centred on blood vessels in the white matter, and hyphae characteristic for *Aspergillus* sp. in blood vessel walls. In the third animal, which had an *Aspergillus*-infection-associated otitis media (see below), *Aspergillus*-like hyphae were detected at the edge of the tissue section of cerebellum, without associated pathological changes.

Viral infection (n=1)

Histology

Brain lesions associated with virus infection were detected in one case. No gross changes were seen in the brain, but histologically in the cerebrum, there was a locally extensive area of increased density of nuclei in the grey matter. In this area, there were perivascular aggregates of lymphocytes and randomly scattered neutrophils in the neuropil. In multiple neurons, the cytoplasm was eosinophilic and the nuclei had large amphophilic inclusion bodies and marginated chromatin. A novel herpesvirus, tentatively named *Phocoena*

phocoena herpesvirus type 2, PPHV-2, was identified in brain samples of this animal. The lesion was diagnosed as a subacute, locally extensive, lymphocytic encephalitis with neuronal necrosis and intranuclear inclusion bodies. The findings of this case have been published previously [23].

Inflammation of unknown aetiology (n=3)

Gross lesions:

No abnormalities detected.

Histology

Three harbour porpoises had brain lesions, for which no aetiology was found. None of these three animals had gross brain lesions, only histological lesions. In the first case, these were diagnosed as moderate, subacute, diffuse, lymphocytic encephalomyelitis, with multifocal gliosis, perivascular lymphocytic cuffing, oedema, and (in the cerebellum) loss of Purkinje cells. In the second case, they were diagnosed as mild, subacute, multifocal, polioencephalitis, and were characterized by the presence of small aggregates of nuclei centred on neurons (neuronophagia) in the cerebrum. In the third case, they were also limited to cerebrum and diagnosed as moderate, subacute, focal, lymphocytic meningo-encephalitis, with neuronophagia.

Beside the six animals described above, CNS involvement was suspected in two more animals. Both animals displayed clinical nervous signs. No gross or histologic lesions were observed in the samples of either animal. One animal had an *Aspergillus fumigatus* infection of the respiratory tract and had epileptic seizures. The other animal had herpesvirus DNA isolated from the brain and cornea and made abnormal uncoordinated swimming movements and had a vertical nystagmus of the right eye.

Liver pathology

Non- inflammatory lesions

In the first case, a pregnant animal with a 60-cm-long male foetus, the liver had an irregular surface and rounded edges. It was diffusely yellow-pale tan and had an increased lobular pattern, both on surface and on section. Histologically, there were areas of necrosis around hepatic venules, characterized by an outer zone of shrunken hypereosinophilic hepatocytes with small dark purple nuclei and a central zone of separated hepatocytes with indistinct cell borders and pale or absent nuclei (karyolysis). Hepatocytes throughout the tissue section had abundant lipid vacuoles in the cytoplasm. These lesions were diagnosed as marked, acute, periacinar hepatic necrosis and moderate, diffuse hepatic lipidosis. The aetiology was not determined, but the lesions resemble those of fatty liver syndrome in cats and equine hyperlipemia in horses (references).

In the second case, a neonate, the liver bulged and had rounded edges. Histologically, the hepatocytes throughout the tissue section contained large smooth-edged cytoplasmic vacuoles, some of which had coalesced into large multilocular vacuoles. In the renal cortex of the same animal, the tubular epithelial cells throughout the tissue section had

similar cytoplasmic vacuoles. Kidney and liver weights were not exceptional compared to other animals of similar length. The aetiology was not determined, but the lesions were suggestive of lysosomal storage disease (e.g., glycogen storage disease type 1) or intoxication (e.g., Swainsona).

Bacterial infection

Liver lesions associated with a bacterial infection were observed in one animal, a juvenile that had bacterial disease in multiple other organs as well as, terminally, a bacterial sepsis. Grossly in the liver, there were two well-demarcated abscesses of 1 cm diameter and filled with yellow-green viscous fluid. Histologically, there were two coalescing abscesses, with a thick wall of fibrous connective tissue around a core of many neutrophils and a rare trematode egg (*Campula oblonga*). A pure culture of *Streptococcus dysgalactiae* was isolated from an abscess sample.

ORGAN SIZES AND WEIGHTS (RELATIVE TO BODY LENGTH) IN RELATION TO LESIONS OBSERVED

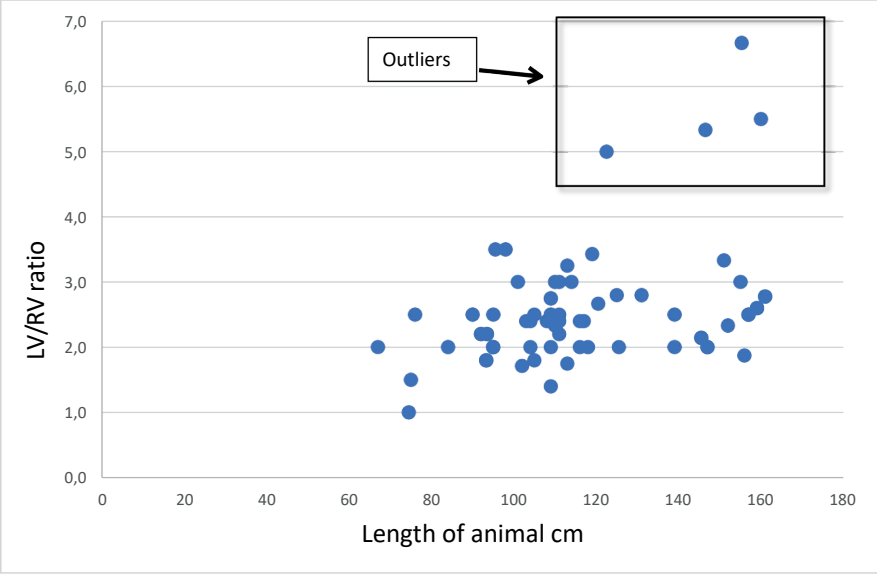
The R^2 was low for spleen (0.17), ratio left ventricle width vs right ventricle width 0.18), ovary (0.34), and left ventricle width (0.42) indicating a poor relation between body length and variable measured. R^2 was intermediate for brain (0.57) and adrenal weights (0.65). R^2 was high for liver (0.77), lung (0.81), kidney (0.83), heart (0.88) and body weight (0.91). For raw data please see Additional file 2.

Comparing highest and lowest 5 % organ sizes (in relation to body length) or the three cases with the highest weight and the three cases with the lowest weight, to diagnosis made indicated: low body weight coincided with findings of emaciation (3 out of 3) and high body weight with findings of good nutritional condition (3 out of 3); high brain weight coincided with diagnoses of encephalitis (2 out of 3); high lung weight coincided with pulmonary congestion (3 out of 3), see also table 8 (incidental diagnoses); low liver weight coincided with cholangitis (2 out of 3); and high adrenal gland weights coincided with hyper- and hypo- plasia of the adrenal gland (2 out of 3). Detailed data are available in excel file Graphs and raw data of organ weights and sizes in additional file 3.
The relation between body length and heart ventricular wall width ratio (L/R) showed four clear outliers out of sixty measurements (see figure 1).
The four cases of an increased LV width/ RV width ratio were all due to a decreased right ventricular wall width. Two juveniles and two adults were involved. All four animals were infected with *P. inflexus* and had pulmonary vasculature lesions ranging from an obstructive thrombus in a pulmonary artery to hyperplastic diffuse chronic vasculitis. In two cases, death was sudden and unforeseen. In one of these two cases, no cause of

death was found by autopsy.

The likelihood that all animals with a high LW width/ RV width ratio were randomly chosen from the population and all had histologic lesions in the vasculature was not significant ($p > 0.05$, Fisher test).

Figure 1 Ratio Left cardiac ventricular width over right cardiac ventricular width in relation to body length



Observations on right ventricle wall thinning

We observed right ventricular wall thinning in four harbour porpoises (additional file Figure 1). The circumstantial facts were suggestive of pulmonary hypertension caused by *P. inflexus* infection of the pulmonary vasculature, leading to dilated cardiomyopathy of the right ventricle. Statistical significance of the association of right ventricular wall thinning and histologic lesions of pulmonary vasculature was weak ($p= 0.16$ for adults and $p=0.08$ for adults, Fisher test), possibly due to small sample size. No supporting evidence exists from other species with a likewise reaction of the right ventricle to pulmonary hypertension. The connection between right ventricular dilation and infection of the pulmonary vasculature by *P. inflexus* therefore remains rather speculative. If future research opportunities present it would be interesting to do extensive diagnostics on harbour porpoise hearts to investigate this relationship.

Table 1 Individual animals with their lesions distributed into lesions which contributed to stranding, did not contribute to stranding or were acquired after stranding, with comment interpreting the severity of lesions.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP030320	• Oral cavity: ulcerative stomatitis, necrotizing, multifocal, acute, moderate. • Penis and preputium: balanoposthitis, suppurative, ulcerative, acute, severe. 3) Incidental diagnoses: • Trachea, bronchi: nematode infection (probably Stenurus minor) • Lung: pulmonary oedema, diffuse, acute, moderate. • Lung: pulmonary congestion, diffuse, acute, moderate. • Oesophagus: ulcerative oesophagitis, multifocal, acute, mild. • Stomach, first section: nematode infection. • Stomach, third section: gastritis, granulomatous, focal, chronic, probably associated with a trematode infection (Pholeter gastrophilus). • Bile ducts: cholangitis, fibrosing, diffuse, chronic, moderate, associated with trematode (Campula oblonga) infection. • Ear, bulla tympanica: nematode infection (probably Stenurus minor). • Skin: ulcerative dermatitis, multifocal, chronic, mild. • Spleen: splenic atrophy, marked.	General: emaciation, severe.		This carcass of a juvenile male harbour porpoise was in poor nutritional condition and freshly dead. The most severe lesion observed at gross necropsy was the severe emaciation. In absence of an obvious underlying cause, lack of food (simple starvation) seems to be the most likely cause. However, further laboratory analyses will be performed to rule out the presence of underlying disease. The differential diagnosis for ulcerative stomatitis, oesophagitis, and balanoposthitis includes herpesvirus and morbillivirus infections. The parasitic infections in lungs, bile ducts, stomach, and ears are common in harbour porpoises and probably had little effect on this animal's health.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP030405	• Lung: pulmonary oedema, diffuse, acute, moderate. • Lung: pulmonary congestion, diffuse, acute, moderate. • Pneumonia, granulomatous, focal, chronic, mild, associated with small lungworm infection • Alveolitis, fibrinous, multifocal, acute, mild. • Tracheitis, suppurative, superficial, diffuse, acute, very mild • Nematode infection (small lungworm) of the alveolar lumina. • Lymph nodes, tracheo-bronchial and lung associated: lymphadenopathy, mild. • Ear, bulla tympanica: nematode infection (probably Stenurus minor). • Dermatitis, superficial, multifocal, mild	Cornea and conjunctiva, bilateral: keratoconjunctivitis, ulcerative, bilateral, chronic, severe. Right eye: keratitis, fibrosing, diffuse, chronic, marked, associated with corneal perforation Pneumonia, pyogranulomatous, multifocal, chronic, severe, associated with bacterial infection.		This carcass of a juvenile female harbour porpoise was in good nutritional condition and freshly dead. The most severe lesion observed at gross necropsy was the bilateral keratoconjunctivitis. No gross lesions were observed in the central nervous system to explain the nervous signs displayed during rehabilitation, but such lesions often only are visible microscopically. The differential diagnosis for ulcerative keratoconjunctivitis includes herpesvirus infection. The severity of the pulmonary lesions was difficult to assess macroscopically. Upon histology these lesions were assessed as relevant.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP031124.2	• Pulmonary oedema acute severe diffuse • Pulmonary hypostatic congestion focal mild • Oral mucosal haematoma focal mild • haemo/hydro pericardium acute mild • Renal pelvic mineralization, multifocal, mild	Emaciation		This female juvenile harbour porpoise was found on the tenth of March 2003 on the beach at Cadzand (Zeeland). After three days of intensive care the animal died. The last 36 hours its condition worsened, i.e. the animal started to vomit and became progressively subdued and was not able anymore to swim without human support. On necropsy the most significant finding is a marked general emaciation. The blubber layer is extremely thin, there is no fat around the internal organs and the back around the dorsal fin is markedly concave. In the pericardium 10 cc of red watery fluid is found. The significance of this finding is unclear. A disease that either uses a lot of energy or inhibits sufficient energy uptake by interfering with digestion or food uptake can cause emaciation. Harbour porpoises are weaned between 8 and 12 months after birth. This is a crucial period where they are extra vulnerable and have to prove they can be self-supporting. Thus, emaciation can also be caused by lack of foraging capabilities. Finally, emaciation can be caused by a general lack of food. In this animal the cause for emaciation was not evident from the macroscopic necropsy. There was no evidence of pneumonia or aspiration of food remains.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP040324	• Tracheitis, lymphocytic, diffuse, chronic, moderate, with epithelial hyperplasia and squamous metaplasia. • Bronchitis, lymphocytic, diffuse, chronic, moderate, with epithelial hyperplasia and squamous metaplasia. • Bilateral general pulmonary congestion, mild • Infestation of the upper respiratory tract with large lungworms (T convolutus or P inflexus) Moderate chronic, bilateral multifocal. • Wound in front of dorsal fin: Fasciitis and panniculitis, focal, moderate, chronic, with formation of fibrovascular tissue. • Tracheo-bronchial and mesenteric lymph nodes: Lymphoid hyperplasia, benign. • Tracheo-bronchial and mesenteric lymph nodes: lymphadenitis, eosinophilic, diffuse, chronic, mild. • Two ulcers on mucosa of medial rostral mandible, multifocal, chronic and mild • Ulcer on right side inside genital slit, focal, chronic mild • Pancreatic duct hyperplasia, diffuse, chronic, marked. • Duodenal ampulla: abscess, associated with parasite infection (likely Pholeter gastrophilus).	• Bronchopneumonia histiocytic, suppurative, multifocal, chronic-active, marked, associated with lungworm infection. • Cerebrum: meningitis, non-suppurative, segmental, chronic, moderate. • Cerebrum: neuronal necrosis, segmental, moderate	• Bilateral general pulmonary oedema, acute and severe.	This carcass of a subadult male harbour porpoise was in poor to moderate nutritional condition and freshly dead. The most significant lesions were a multifocal puro granulomatous pneumonia and moderate neuronal necrosis and meningitis of the cerebrum associated with herpes virus DNA. The foci in the lungs were mostly associated with the remnants of large lungworms (length 10 – 30 cm). The lung worm infestation was bilateral and moderate to severe (100-1000 lungworms per lung). The cause of the large wound in front of the dorsal fin was unclear but trauma seems a likely cause. The wound had not penetrated the blubber layer. The surface consisted of repair tissue which means the wound was at least several days old. The cause of the ulcers in the oral cavity and the genital slit is not known.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP040517	• Moderate chronic corneal ulcus on left eye • Pulmonary-associated lymph node: lymphoid hyperplasia, moderate • Pulmonary blood vessels: nematode infection • duodenal submucosal abscess, focal, chronic, mild. • colonic crypt abscesses, mild • Mild chronic ulcus on inner side left genital slit • Mild chronic ulcus on rostral tip of palatum durum.	• bronchopneumonia suppurative, multifocal, chronic-active, associated with lungworm larvae. • pneumonia, necrotizing, focal, acute, mild, associated with Aspergillus fungal hyphae		This male juvenile harbour porpoise in moderate condition was euthanized after repeated epileptiform attacks. The primary cause of his clinical symptoms is most likely a subacute severe diffuse pneumonia. The general pulmonary oedema might have resulted from the euthanasia. Alternatively, the oedema might be the result of heart failure or damaged alveolar type 1 epithelium due to systemic toxins, endotoxins or shock like states. The interstitial emphysema indicates the porpoise had experienced severe respiratory difficulties. The gas in the lymph vessels indicates the emphysema was not peracute and did not result from the euthanasia. No abnormalities were detected in the central nervous system upon macroscopic examination which could have been responsible for the epileptiform attacks.
PP040526	• Patent urachus • Muscular petechiae multiple, localized acute, moderate. • Hepatic lipidosis, diffuse, mild. • Liver congestion, diffuse mild and acute. • Subepithelial oesophageal congestion, diffuse, acute mild • Mucosal fore stomach congestion, diffuse acute mild • Meningeal congestion, diffuse, acute mild • Patent ductus botallicus	Interstitial pneumonia, suppurative, multifocal, acute, moderate.	• Pulmonary oedema, acute, diffuse, bilateral, severe	This neonatal male harbour porpoise most likely has died of suffocation due to severe pulmonary oedema and the interstitial pneumonia. Neurogenic shock resulting from transportation stress is a cause that has to be considered. The petechiae found on the thoracic wall support this speculation. The significance of the congestion in oesophagus, fore stomach and meninges is uncertain. The patent urachus did not lead to a bacterial invasion and sepsis, based on the negative findings of the bacterial examination.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP040627	• Bronchopneumonia, histiocytic, suppurative, diffuse, chronic, mild. • Fore stomach, glandular stomach: foreign body (both stomachs five ml • of sand) • Omphalitis, haemorrhagic, suppurative, focal, acute, mild. • Moderate diffuse acute liver congestion		Acute moderate diffuse bilateral pulmonary oedema.	This male neonatal harbour porpoise was euthanized within twelve hours of being admitted in the rehabilitation centre. No evident cause was found for the severe clinical symptoms that were seen. The sand in the fore stomach and the glandular stomach might have been caused by a rough stranding on a sandy beach. The pulmonary oedema and the liver congestion are normally seen in carcasses of euthanized animals. The omphalitis and pulmonary infection seem clinically insignificant.
PP041215	• Pulmonary blood vessels: vasculitis, hyperplastic, diffuse, chronic, moderate, associated with nematode infection. • Keratitis focal chronic moderate • Lung: nematode infection	• •pneumonia, necrotizing, suppurative, locally extensive, acute, marked, associated with bacterial infection • bronchitis, bronchiolitis and alveolitis, granulomatous, suppurative, multifocal, chronic, moderate, associated with adult and (especially) larval nematodes. • Sepsis		This juvenile male in poor nutritional condition died after two days in the rehabilitation centre. The most significant finding was a focal pneumonia in the right side of the lungs associated with parasites and a bacterial infection which had spread throughout the body (sepsis)

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP050208	• Pulmonary artery: arteritis, proliferative, multifocal, chronic, moderate, associated with nematode infection. • Lung-associated lymph node: lymphoid hyperplasia, moderate. • Lung-associated lymph node: lymphadenitis, eosinophilic, diffuse, subacute, moderate. • Adrenocortical hyperplasia bilateral, chronic, severe • Pancreatic angitis, proliferative, eosinophilic, diffuse, chronic, marked, associated with parasitic infection • Cholangitis, proliferative, eosinophilic, diffuse, chronic, marked, associated with parasitic infection. • Skin lesions, multifocal scars, healed • Skin lesions, multifocal discolourings, chronic mild (epidermal intracytoplasmic eosinophilic inclusions (poxvirus)) • Intestine, hypostatic congestion, mild • Peritoneal cyst, focal mild • Subcutal inflammation multifocal chronic mild • Renal visceral discoulorings one sided multifocal, mild • Urinary vesical wall congestion diffuse, mild • Pulmonary hypostatic congestion • Pulmonary purulent inflammation, focal mild • Bronchiolar parasitic infestation, multifocal, chronic mild	• Bronchopneumonia, mixed, multifocal, chronic-active, marked, associated with two nematode species and suspect microbial infection	• Pulmonary oedema, moderate, acute diffuse. • Pulmonary haemorrhage, focal, acute moderate	This adult female harbour porpoise in good body condition died during a diagnostic bronchoscopy. The most important findings were a bronchopneumonia, possibly caused by a bacterial infection (not cultured) and or a fungal infection (cultured but not clear on histology) and a severe parasitic infestation of the pulmonary vessels and a moderate haemorrhage of the right lung. The parasitic infection might have had a strong negative effect on the perfusion of the lung thus causing hypoxia and in combination with the bronchopneumonia it caused an inability of the animal to dive. If the animal already failed to dive when at sea this could explain all the scars and wounds found at stranding by bird pecking. The existing hypoxia was not helped by the sedation which decreases breathing rate. The haemorrhage and the stress of the intervention adding to the negative existing hypoxia might have caused fatal hypoxia leading to shock or brain damage and apnoea. The large adrenals might have been caused by chronic stress. All other findings are incidental.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP050502	• Bronchitis, lympho-plasmacytic, suppurative, diffuse, subacute, moderate • Lymph node, prescapular (?): Lymphoid hyperplasia, moderate • Pleuritis multifocal unilateral chronic moderate • Pericarditis, focal chronic moderate • Lymfonodular enlargement pulmonary multifocal chronic moderate • Cystitis, eosinophilic, diffuse, superficial, acute, mild. • Mesenteric lymph node: lymphoid hypoplasia, moderate. • Inflammation of the pancreatic duct, lympho-plasmacytic, fibrosing, diffuse, chronic, moderate • Adrenocortical hypoplasia, diffuse, marked, associated with exogenous corticosteroid therapy • Papilloma, with characteristic basophilic intranuclear viral inclusion bodies (herpesvirus) • Gastritis, lympho-plasmacytic, eosinophilic, superficial, multifocal, subacute, mild • Dermatitis, multifocal, suppurative, superficial, acute, moderate, associated with bacterial infection. • Dental abrasion multifocal chronic mild • Mesenterial lipoma's multifocal mild • Hepatitis multifocal chronic mild and associated with the presence of trematodes • Vaginal mucosal lesions multifocal chronic mild • Unilateral corneal oedema focal mild • Dermal papilloma Focal chronic mild • Mammarian cysts multifocal chronic mild • Thrombosis (fundic stomach), focal, reorganized, recanalized • Colitis, eosinophilic, lympho-plasmacytic, superficial, diffuse, chronic, mild.	• Alveolitis, histiocytic, multifocal, chronic, moderate to severe, with abundant foamy material containing small round pink bodies about 0.5 µm diameter • Dermatitis ulcerative multifocal chronic moderate to severe • Panniculitis multifocal mild	• Pulmonary oedema acute diffuse mild • Kidney failure leading to unacceptable clinical symptoms and euthanasia • Nephritis, suppurative, focal, acute, mild	This adult female harbour porpoise in moderate body condition was euthanized after 1 ½ years of treatment at the rehabilitation centre of the Dolfinarium Harderwijk. The animal succumbed to kidney failure, which was most evident in the blood serum values. Urea increased from 18 to 30 mmol/L, creatinine from <27 to 81 µmol/L and sodium from 158,5 to 196,4 mmol/L. The animal suffered from infections in multiple locations, dermatitis, cystitis, nephritis, bronchitis, pancreatic duct inflammation, colitis and gastritis. The dermatitis was the most severe. An ascending cystitis might be responsible for the acute nephritis, which caused severe clinical symptoms. The respiratory tract was despite enduring treatment not free from chronic (bacterial) infection as demonstrated by the chronic pleuritis, miliary abscesses in the rostral left lobe and the suppurative bronchitis. The pulmonary alveolar proteinase which was diagnosed per exlusionem based on histology is in humans known to cause chronic pulmonary infections and may increase the susceptibility of all organ systems to infection due to the failure of granulocyte macrophage colony stimulating factor (GM-CSF) negatively influencing the immune reaction. The applied anti-parasitic treatment was apparently effective only 2 live nematodes were found in the pulmonary venae. The diagnose of pulmonary alveolar proteinase needs to be confirmed by electron microscopy. The genital lesion which had the outward appearance of a papilloma was associated with the presence of herpes virus like particles and a herpes positive PCR

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP050610	• Trachea, bronchus: tracheobronchitis, superficial, chronic, diffuse, with multifocal squamous metaplasia of respiratory epithelium. • Pulmonary parasitic pneumonia multifocal chronic mild • Hepatic congestion diffuse acute moderate • Thyroid gland: hyperplastic goitre or thyroid follicular atrophy (dependent on thyroid weight). • 3) Incidental diagnoses: • Multifocal dermal scarring chronic mild (healed) • Multifocal dermal nodules chronic mild • Lung: small lungworm infection (probably Stenurus minor) in lung parenchyma. • -Pulmonary artery: arteritis, multifocal, chronic, mild, with thrombosis, associated with heartworm infection (probably Torynurus convolutus). • -Oesophageal artery: arterial mineralization. • Pulmonary associate lymph node: lymphoid hyperplasia, diffuse.	Multifocal granulomatous infection of diverse organs and serosa of the thorax chronic severe, associated with Aspergillus infection • Right and left heart ventricle: epicarditis and myocarditis, granulomatous, necrotizing, multifocal, chronic, moderate, associated with Aspergillus infection. • Granulomatous myocarditis multifocal chronic severe • Pyothorax bilateral mild • Lung: bronchopneumonia, necrotizing, haemorrhagic, granulomatous, multifocal or diffuse, chronic, marked, associated with the presence of Aspergillus hyphae. • Pleura: pleuritis, granulomatous, multifocal or diffuse, moderate or marked, associated with the presence of Aspergillus hyphae. • Granulomatous mediastinitis multifocal chronic moderate • Vertebral osteomyelitis focal chronic moderate	• Pulmonary oedema general acute severe • Vertebra: osteomyelitis, pyogranulomatous, focal, chronic, marked, with exophytic bone formation, associated with bacterial infection. • -Skeletal muscle at injection site: muscle necrosis and haemorrhage, focal, acute, marked, associated with imipenem injection.	COMMENTS: This juvenile female harbour porpoise stranded in moderate body condition. The most significant finding was a multifocal granulomatous infection of multiple organs and serosa of the thorax, associated with an Aspergillus fumigatus infection. The infection likely originated from the lungs and spread per continuitatum to the pleura and pericardium. A possible osteomyelitis of the caudal vertebral column might have caused deterioration of the condition and resistance of the harbour porpoise or might have occurred as a result of the disease process causing the animal to be less rapid and evasive of predators.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP050825.1	• Vasculitis, granulomatous, with endothelial hyperplasia, fibrosis, and thrombosis, moderate, associated with nematode infection. • Bronchitis, lymphoplasmacytic, locally extensive, chronic, moderate, associated with large lungworm infection. • Pulmonary blood vessels: vasculitis, suppurative, associated with bacterial infection superimposed on a nematode infection. • Pulmonary lymph node: lymphoid hyperplasia, moderate. • Dermal lesion focal mild • Dermal trauma lesions multifocal acute mild • Gastritis multifocal chronic mild	• Pneumonia, granulomatous, multifocal, chronic, moderate, associated with large lungworm infection • Pneumonia, histiocytic, eosinophilic, locally extensive, chronic, moderate, associated with small lungworm larvae.	• Pulmonary oedema	This juvenile male harbour porpoise in good nutritional state died during transport to the rehabilitation centre. Cuts on the tail fluke and abundant pulmonary oedema were found and are in accordance with bycatch. Empty stomachs, absence of chyle in the mesenteric lymphatic vessels, are not in accordance with bycatch. The severe pulmonary oedema is the probable cause of death. Possibly the oedema was caused by increased oxygen requirement due to transport stress in combination with poor ventilation and perfusion due to severe parasite infestation of bronchi and pulmonary arteries plus abnormal posture. The good body condition argues however against continuous respiratory distress as the harbour porpoise had been able at least until very recently to feed itself adequately.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP050825.2	• Pulmonary artery: arteritis, proliferative, multifocal, chronic, associated with nematode infection (likely Pseudalius inflexus). • Phlebitis verminous multifocal chronic mild • Gastric granuloma, focal, chronic, mild, associated with encapsulated parasite (likely Pholeter gastrophilus).	• Encephalitis, lymphocytic, locally extensive, subacute, with neuronal necrosis and intranuclear inclusion bodies (herpesvirus). • Bronchopneumonia, pyogranulomatous, multifocal, chronic, associated with nematode infection (probably Stenurus minor).	• Pulmonary oedema diffuse acute severe	This female juvenile harbour porpoise in poor nutritional condition was euthanized due to aggravating dyspnoea. The most important morphologic diagnosis is the pyogranulomatous bronchopneumonia, which in itself was probably not severe enough to cause the apparent dyspnoea, the arteritis of the pulmonary artery which may have caused severe perfusion ventilation mismatch which might explain the dyspnoea. The third important morphological diagnosis was encephalitis which was likely caused by herpesvirus. Herpesviruslike particles were observed by electron microscopy in lesions of the cerebrum and herpesvirus presence was confirmed by polymerase chain reaction on samples of the cerebrum. Alternatively, the dyspnoea was a behavioural aberration caused by the herpesvirus encephalitis. Shivering may have been caused by the need of the body to heat up or alternatively by the encephalitis. Shivering was also noted in a white beaked dolphin with morbillivirus encephalitis. The pulmonary oedema is most likely to have been caused by the euthanasia.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP051106	• Trachea: tracheitis, lymphoplasmacytic, superficial, diffuse, subacute, moderate. • Bronchus: bronchitis, lymphoplasmacytic, superficial, diffuse, subacute, moderate. • Bronchiole: bronchiolitis obliterans, focal, chronic, marked, associated with nematode infection (possibly Pseudalius inflexus). • Adrenal cortex: adrenocortical haemorrhage, multifocal, acute, moderate. • Forestomach: gastritis, ulcerative, multifocal, acute, mild to marked, associated with bacterial and parasitic infection. • Fundic stomach: gastritis, fibrosing, focal, chronic, associated with trematode infection (probably Pholeter gastrophilus). • Bile duct: cholangitis, lymphoplasmacytic, locally extensive, chronic, marked, with epithelial hyperplasia and fibrosis, associated with trematode infection (probably Campula oblonga) • Anaemia diffuse moderate • Dermal lesions multifocal acute mild • Dermal lesions multifocal chronic mild	• Exhaustion and emaciation • Pulmonary blood vessel: thrombosis, partly to completely obliterative, chronic-active, marked, associated with large nematode and (focally) fungal infection. • Pulmonary blood vessel: vasculitis, hyperplastic, lymphoplasmacytic, diffuse, marked, chronic, associated with large nematode infection (possibly Pseudalius inflexus). • Multifocal atelectasis subacute to chronic moderate • Lung: pulmonary oedema and congestion, diffuse, acute, moderate		This pregnant female harbour porpoise in poor body condition probably died due to exhaustion and emaciation. The most relevant lesions were the subacute to chronic diffuse pneumonia and bronchitis obliterans caused by nematode infection (Pseudalius inflexus) plus the marked vasculitis and obliterative thrombosis of the pulmonary vasculature, associated with Pseudalius inflexus infestation and fungal infection. The multiple gastric ulcers will have caused chronic blood loss and the observed anaemia will have been partly due to these ulcers. Toxic substances from the chronic inflammation of the lungs might also have contributed to the observed anaemia. The anaemia in combination with the small function lung capacity left must have caused poor oxygenation of all organs. The multifocal hepatitis is probably due to a C. oblongata infection although no trematodes could be found. The relevance of the pancreatic cyst is unclear. In humans a cyst can be caused by acute or chronic inflammation or be a benign or malignant tumour. Histology might give more clues towards the interpretation of this lesion. The dermal lesions are insignificant. The linear scar on the right side of the head could be caused by the head of the porpoise being stuck in a net but this is very speculative.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060220	• Pulmonary artery: thrombosis, chronic, moderate • Keratitis focal acute mild • Hepatic lipidosis diffuse acute to chronic moderate associated with possible physiologic gravid hepatic lipidosis • Hepato cholitis chronic multifocal to coalescing mild • Pneumonia, chronic-active, multifocal, mild. • Dermatitis, suppurative, superficial, focal, acute, mild. • Skin: dermal haemorrhage, focal, subacute, mild. • Fundic stomach: gastric erosion, focal, acute, mild	• Cardiac malformation unilateral acute to chronic severe • Skeletal muscle degeneration, diffuse, subacute, marked.		This gravid female harbour porpoise died after 4 days in a rehabilitation centre. The main findings were a fatty liver and an abnormal heart with abnormal ratio of left and right ventricle thickness and a right ventricle which had less consistency than normal. A fatty liver is physiologic in gravid dolphins but could also be a sign of acetonemia. Acetonemia would possibly explain the observed spasms and the poor coordination during swimming. However, liver enzymes were not as elevated as in most other strandings and there was no typical smell of ketone bodies which has been observed several times in stranded harbour porpoises. Heart failure might have been responsible for a deteriorated fitness and thus less ability to forage, hence the poor body condition. It might also explain the rather unexpected death of the animal.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060227	• Tracheobronchial and mediastinal lymph node: lymphoid hyperplasia, diffuse, moderate to marked • Keratitis, suppurative, diffuse, chronic-active, marked, with vascularisation, pigmentation, and retrocorneal membrane • Cellulitis multifocal acute to chronic severe • Pulmonary inflammation chronic focal mild • Cholangitis, lymphoplasmacytic, locally extensive, chronic, marked, with bile duct hyperplasia and fibrosis, associated with trematode (Campula oblonga) infection. • Pulmonary vein: phlebitis, lymphoplasmacytic, diffuse, chronic, very mild, associated with nematode infection • Blubber: panniculitis, histiocytic, multifocal, chronic, mild. • Lung: pulmonary granuloma, chronic, mild, associated with lungworm infection. • Lung: bronchitis, lymphoplasmacytic, diffuse, chronic, mild. • Right epididymis: Epididymal cyst, unilateral, chronic, marked.	• Lung: bronchopneumonia, suppurative, locally extensive, acute, moderate • Cerebrum: encephalitis, lymphocytic, diffuse, subacute, moderate, with multifocal gliosis, perivascular cuffing, and oedema. • Cerebellum: meningo-encephalitis, lymphocytic, subacute, moderate, with multifocal gliosis and loss of Purkinje cells. • Cervical spinal cord: myelitis, lymphocytic, diffuse, subacute, moderate.	• Pulmonary oedema diffuse acute marked • Pulmonary congestion diffuse acute moderate	This male harbour porpoise in good nutritional condition died after 16 days in a rehabilitation centre. The most significant lesion is the encephalitis. It is restricted to the grey matter (so, polioencephalitis), and is characterized by neuronal necrosis, foci of gliosis and oedema, and perivascular lymphocytic cuffing. This is typical of a viral or protozoal infection, and because no zoites or cysts are seen, protozoal infection is less likely. A viral infection may be responsible for the polioencephalitis. The pulmonary pathology will have contributed to the demise of the animal.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060301	• Corneal perforation, bilateral, with iris prolapse, severe diffuse suppurative keratitis, corneal vascularization, and corneal pigmentation. • Hepatic atrophy, diffuse, marked. • Hepatic bile duct cysts, multifocal, coalescing • Dermatitis, hyperplastic, locally extensive, chronic, moderate, with intracytoplasmic inclusion bodies (poxvirus). • Dermatitis, suppurative, multifocal, superficial, acute, mild • Bronchopneumonia, suppurative, multifocal, peracute, mild, associated with bacterial infection • Small intestine inflammation focal acute mild. • Bronchiolar infection with nematode parasite • Pulmonary granuloma, focal, chronic, mild, associated with nematode parasite infection • Perivascular oedema focal acute mild	• Pancreatic duct hyperplasia, diffuse, chronic, marked.	• Pulmonary oedema diffuse acute severe • Pulmonary congestion unilateral hypostatic • Pulmonary atelectasis post mortem diffuse	This juvenile female harbour porpoise was euthanized after a six day stay in a rehabilitation centre. The main clinical symptom was severe emaciation upon arrival and weight loss despite a large amount of food intake during her stay. During her final day breathing frequency increased significantly indicating shortness of breath. No chylum was observed in the mesenteric lymph vessels despite digested food being present in the intestines. Total protein level in the serum dropped from 66 to 31 gr/l, which is dramatic. Malabsorption in the intestine thus appears the most likely cause for the continuing weight loss. Malabsorption might have been caused by absence of pancreatic enzymes due to pancreatic duct obstruction. This obstruction is speculatively caused by the formation of hepatic cysts due to congenital ductal plate malformation. Malabsorption led to hypo-proteinemia and as sequel oedema around vessels on the heart and severe oedema in the lungs, which caused shortness of breath. The pneumonia, which was observed, was mild and insignificant as a cause of clinical symptoms.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060327.1	• Pneumonia, chronic-active, multifocal, mild • Lymphadenopathy multifocal subacute to chronic moderate (pulmonary Lnn) • 3) Incidental diagnoses: • Aortic nodule associated with the remains of the ductus of Botallicus	• Pectoral fin traumatic lesion subacute to chronic focal severe	• Pulmonary oedema diffuse acute severe	This juvenile female harbour porpoise in good nutritional condition was found with a partly amputated right pectoral fin. The trauma on the fin had partly healed and the fin was not inflamed. The straightness of the cut and the absence of further trauma on the pectoral fin makes it likely a knife rather than a bite wound caused this trauma. The observed pneumonia was not severe enough to have caused significant clinical problems. The digestive tract was empty possibly because the animal was not capable anymore of hunting after the trauma had occurred.
PP060327.2	• Dermal ulceration multifocal chronic mild • Dermatitis, suppurative, multifocal, acute, mild.	• Pulmonary empyema multifocal acute moderate • Smooth muscle hyperplasia, multifocal, chronic, marked, possibly associated with infection with nematodes (probably Torynurus convolutus). • Pulmonary abscesses, multifocal, chronic, moderate, possibly associated with large and small nematodes (probably Torynurus convolutus, Pseudalius inflexus, and Halocercus invaginatus) and cocci. • Broncho-interstitial pneumonia, histiocytic, multifocal, chronic, mild, associated with adult and larval small nematodes (probably Halocercus invaginatus). • Emaciation chronic severe	• Pulmonary oedema diffuse acute severe	This juvenile male harbour porpoise in very poor nutritional condition died after 8 hours in the rehabilitation centre. The most marked lesions were seen in the lungs, and were associated with nematode infections both of the airways and the blood vessels. In addition, at least one pulmonary abscess had evidence of bacterial co-infection. Together, these lesions could have been responsible for the animal's death.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060327.3	• Oesophageal ulcers multifocal acute to chronic moderate • Gastric ulcer, focal, superficial, chronic, moderate. • Gastritis, suppurative, superficial, acute, mild. • Oral ulcer focal acute to subacute moderate • Lymphadenopathy multifocal acute to chronic moderate • Pulmonary artery: vasculitis, thrombosing, suppurative, associated with nematode parasites. • Ocular traumatic lesion focal peracute severe • Dermal ulcers multifocal chronic mild • Dermatitis, suppurative, diffuse, acute, moderate. • Hard palate of mouth: ulcer, focal, acute, mild	• Jaundice diffuse subepidermal fat layer and the aorta moderate • Hepatitis, necrotizing, multifocal, acute, marked. • Bile duct hyperplasia, diffuse, chronic, marked. • Interstitial pneumonia, locally extensive, histiocytic, chronic, moderate, associated with unknown organisms.	• Tracheo-bronchitis, lymphoplasmacytic, suppurative, superficial, diffuse, subacute, marked, associated with food aspiration, cocci, and bacilli.	This adult male harbour porpoise in good nutritional condition died after five days in the rehabilitation centre. The main lesions are found in the lungs the most marked lesions in this animal are a tracheo-bronchitis, interstitial pneumonia, and hepatitis. The cause of the tracheo-bronchitis may be aspiration. The cause of the interstitial pneumonia is not clear: part of the lesions may be due to the round intra-macrophagic organisms, but other etiologic agents may play a role because the extent of the pneumonia is greater than the distribution of the organisms. The cause of the hepatitis is not clear. Also, the importance of the hepatitis is not clear: in one section, the area of affected tissue is large, but in another section, it is not. The anaemia might be the result from blood loss from the stomach and oesophageal ulcers and from the lungs damaged by the parasites. The ocular trauma is caused after the animal has stranded. It is not clear if one aetiology is present for the mouth stomach and oesophageal ulcers, like acid reflux from the forestomach. The fore stomach smell strongly of acetone, which has been noted before with animals with an ulcer associated with parasites. Possibly the caustic effect of acetone is causing the ulcers due to reflux. The aetiology of the biliary tract infection is unclear. Usually parasites can be found in the biliary tracts but this was not so in this animal. The macroscopically observed jaundice does not correspond to the bilirubin values measured in the blood.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060327.4	• Hypostatic pulmonary congestion multifocal mild • Dermatitis focal chronic mild		• Pulmonary oedema diffuse acute and severe.	This juvenile male harbour porpoise in good nutritional condition died after 3 days in the rehabilitation centre in the Dolfinarium in Harderwijk. The clinical symptoms indicate gastro-intestinal problems (vomiting, stasis of gastric content) and problems of the respiratory tract (forced breathing, high breathing frequency, exudates during expiration from blowhole). Multiple organ systems might be affected by sepsis or toxic substances from the infection of the respiratory tract might have adverse effects on the digestive tract. The lack of macroscopic lesions in the lungs is remarkable considering the clinical symptoms. The influence of the opiate administered on respiration has to be considered. No respiratory depression was observed.
PP060501	• Traumatic dermatitis multifocal acute mild • Subperitonal haemorrhage multifocal acute mild • Ulcerative keratitis focal subacute moderate • Pulmonary blood vessel: nematode infection.	• bronchopneumonia, suppurative, locally extensive, acute, moderate, associated with nematode larvae (lungworm larvae).	• Fundic stomach: Gastric epithelial erosion, focal, acute, mild. • Adrenocortical haemorrhage, multifocal, acute, moderate.	This juvenile female harbour porpoise in good nutritional condition died of an undetermined cause. The lungworm-larvae-associated bronchopneumonia may be the most significant diagnosis. The gastric erosion and adrenocortical haemorrhages are suspected to have occurred peri-mortally.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP060524	• Bronchopneumonia, granulomatous, multifocal, chronic, moderate, associated with adult and larval nematodes (small lungworms). • Ulcerative gastritis (cardiac) focal chronic moderate associated with nematode and bacterial infection • Ulcerative keratitis fibrosing focal subacute to chronic severe • Endophthalmitis, fibrosing, chronic, marked • Atrophic bulbi • Tracheo bronchial and prescapular lymph nodes: lymphadenopathy subacute to chronic moderate. • Subcutaneous oedema focal acute to subacute moderate • Pulmonary atelectasis multifocal acute mild • Subpleural haemorrhages acute diffuse mild • Pneumonia multifocal chronic mild associated with nematode infection • Pneumonia focal chronic mild. • Fundic section of stomach: gastritis, granulomatous, multifocal, chronic, mild, probably associated with parasite infection • Dermal lesions acute and chronic multi focal mild • Mesenteric lymph node: lymphadenitis, granulomatous, multifocal, chronic, mild, probably associated with parasitic infection.	• Pachymeningitis, necrotizing, suppurative, locally extensive, acute, marked, associated with fungal hyphae (Aspergillus sp.). • Cerebellum: panencephalitis, pyogranulomatous, haemorrhagic, necrotizing, locally extensive, associated with fungal hyphae (Aspergillus sp.) and mixed bacterial infection. • Otitis media purulent subacute to chronic diffuse severe • Pia mater of cerebellum: leptomeningitis, suppurative, acute, mild • Pia mater of cerebrum: leptomeningitis, lymphocytic, subacute, mild	• Pulmonary oedema diffuse acute severe	This juvenile female harbour porpoise in good nutritional condition died 9 hours after being admitted into the rehabilitation centre. The main lesions were a severe panencephalitis, pachymeningitis and otitis media caused by Aspergillus sp. infection combined with a mixed bacterial infection. This resulted in marked inflammation and necrosis of the cerebellum and overlying meninges. The cause is unclear, but the presence of multiple pathogens suggests direct introduction from the external environment, e.g., via a perforated eye. The Clostridium perfringens which was cultured form the middle ear and the skull lesion might have been successful in the live animal due to the anaerobic conditions created by the tissue damage and necrosis caused by the other invaders.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP061122.1	• Pulmonary thrombosis, associated with infection with nematode worms (likely Pseudalius inflexus) • Spleen, lymph nodes: increased numbers of hemosiderin-laden macrophages • Dermatitis, suppurative, hyperplastic, chronic, multifocal, mild, associated with Candida sp • Dermal lesions multifocal acute mild • Myositis, lymphocytic, subacute, mild • Corneal oedema diffuse acute mild • Parasitic infestation multifocal chronic mild to moderate	• Bronchopneumonia, pyogranulomatous, necrotizing, chronic, moderate, associated with Aspergillus sp. Infection • Bronchopneumonia, pyogranulomatous, multifocal, chronic, mild to moderate, associated with lungworm (likely Stenurus minor) infection • Emaciation diffuse chronic severe	• Pulmonary oedema diffuse peracute severe • Gastric impaction acute severe. • Sepsis	This female juvenile harbour porpoise in poor nutritional condition was euthanized. The most significant lesion appears to be the pyogranulomatous bronchopneumonia, associated both with S. minor larvae and Aspergillus sp. infection. The gastric impaction is known to occur in debilitated animals and might be a consequence of malfunctioning of the GI tract due to emaciation or sepsis. Enterococcus faecalis sepsis was demonstrated by bacteriological examination. The organism was cultured exclusively out of the lung, spleen, liver, adrenal, and uterus. A mixed culture was found in the kidneys and the lung associated Ln (together with Enterococcus faecalis).

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP061122.2	• Bronchopneumonia, granulomatous, multifocal, chronic, mild, associated with nematode infection (probably Stenurus minor). • Dermatitis multifocal purulent acute mild • Dermal lesion chronic focal mild • Parasitic infestation multifocal chronic mild to moderate • Oesophageal ulcer, focal, acute, mild • Pancreatic duct hyperplasia, mild	• Sepsis secondary to pneumonia • Bronchopneumonia, suppurative, multifocal, subacute to chronic, severe, associated with Escherichia coli infection • Myositis, suppurative, multifocal, acute, moderate (consistent with Escherichia coli infection). • Fasciitis, suppurative, focal, acute, moderate (consistent with Escherichia coli infection). • Pulmonary thrombosis, organizing, multifocal, chronic, moderate, with nematode (likely Pseudalius inflexus) and bacterial (likely Escherichia coli) infections.		This male juvenile harbour porpoise in poor body condition died in the rehabilitation centre. The most significant lesion was the severe pneumonia. The observed sepsis will have come from the pneumonia. Abscessation might explain the resilience of the E. coli to amikacin treatment. The E. coli also infected the nematode-induced pulmonary thrombi, likely resulting in septic emboli that spread to other parts of the body. The use of prednisolone can have facilitated the occurrence of sepsis.
PP061123.1	• Hepatic congestion acute diffuse moderate	• Pulmonary oedema, diffuse, acute, marked. • Renal tubular necrosis, multifocal, acute, moderate, associated with hyaline granular casts (myoglobin?).		This juvenile female harbour porpoise in good nutritional condition died during transport. The most significant finding was the acute pulmonary oedema. Together with the suspected myoglobin casts and tubular necrosis in the kidney this speculatively points to capture myopathy leading to heart failure and death.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP070221	• Cholangiohepatitis, diffuse, fibrosing, chronic, marked, associated with trematode infection, (Campula oblongata) • Dermatitis, focal, suppurative, acute, mild, with suspect poxvirus inclusions. • Skin cut, chronic, with focal suppurative dermatitis and focal fibroplasia (physical trauma). • Dermatitis, suppurative, focal, acute, associated with bacterial infection and cestode infection. • Pulmonary haemorrhage focal subacute moderate. • Pulmonary oedema diffuse acute mild • Pyloric stomach: gastritis, focal, fibrosing, ulcerative, chronic, moderate, associated with trematode infection (Pholeter gastrophilus). • Bronchus: lungworm infection (probably Halocercus invaginatus and Stenurus minor) • Pulmonary artery: arteritis, fibrosing, diffuse, chronic, mild to severe, associated with nematode infection (Torynurus convolutus). • Pleural oedema focal acute mild • Emphysema, multifocal, mild • Splenic lymphoid hyperplasia, moderate	• Bronchopneumonia multifocal, lymphocytic and eosinophilic, chronic, moderate, associated with nematode infection., (probably Halocercus invaginatus and Stenurus minor) • Bronchopneumonia multifocal, suppurative, acute, moderate, associated with bacterial infection.		This juvenile male harbour porpoise in poor nutritional condition died 20 minutes after his arrival in the rehabilitation centre. The primary diagnosis was pneumonia. The pneumonia had a chronic component associated with nematode infection and an acute component associated with a bacterial infection. Possibly the parasitic infection may predispose for a bacterial infection. The partial occlusion of the pulmonary arteries by nematodes might be more significant then hitherto assumed in disturbing the perfusion ventilation relation of the lung tissue and needs further examination in live animals.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP070317	• Bronchi: nematode infection (probably Halocercus invaginatus) diffuse chronic marked • Pneumonia, pyogranulomatous, multifocal, chronic, mild • Pulmonary oedema, diffuse, acute, mild. • Interstitial pneumonia, granulomatous, focal, chronic, mild • Parasitic infection of the alveoli (probably Stenurus minor). • Bronchopneumonia, lymphoplasmacytic, diffuse, chronic, mild, associated with bacterial infection (probably Pseudomonas aeruginosa) • Pulmonary abscess focal chronic mild associated with trematode (?) infection • Haemorrhages, subpleural, pulmonary and costal, multifocal, acute, mild. • Cholangitis, proliferative, chronic, marked, associated with Campula oblonga infection. • Pulmonary artery: nematode infection (probably Torynurus convolutus) • Thyroid congestion, diffuse, acute, marked.	• Hepatic necrosis, peri-acinar, extensive, acute, marked associated with a moderate icterus • Hepatic lipidosis, diffuse, acute, moderate		This adult female gravid harbour porpoise was in good nutritional condition and freshly dead upon necropsy. The death of this animal is most likely related to the severe hepatic necrosis and lipidosis, which is associated with the generalized icterus (visible most clearly by the yellow colour of the intima of the aorta and pulmonary trunk). The liver disease fits with the results of clinical chemical analysis, which show strongly increased blood concentrations of liver enzymes (ASAT, ALAT, LD). The cause of the hepatic necrosis is not clear. It has a resemblance to fatty liver syndrome in cats and equine hyperlipemia in horses. Both diseases are conditions in fat animals that suffer some kind of stress, and animals dying from this disease develop severe hepatic lipidosis. In horses, pregnant or lactating mares are predisposed. In both cats and horses the pathogenesis is obscure. Histological evaluation of the liver may help to make a more precise diagnosis. Severe leukopenia was observed before the rise of liver enzymes. The leukopenia is due to a marked inflammatory reaction of which the location remains obscure. Toxins from the inflammation site in combination with the existing moderate lipidosis are likely to have caused the hepatic necrosis, which ultimately caused the death of the harbour porpoise. Although the large lungworms in this porpoise occluded a large part of the primary bronchial lumina and would likely affected have affected its endurance, I doubt that this infection played an important role in its death, since the infection was chronic and the porpoise was in good nutritional condition. The small nodules in the lung were likely remnants of small lungworms and of little consequence. The pleural haemorrhages likely occurred just before death, perhaps due to excessive respiratory movements. The trematode infection of the bile ducts was chronic and the associated lesions were mild. No clear lesions were seen in association with the heartworm infection. This adult female was gravid with a 60-cm-long male foetus. Foetus, uterus, and placenta appeared normal.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP070328	• tracheo-bronchitis, lymphoplasmacytic, erosive, diffuse, chronic, mild. • lymphonodular hyperplasia (tracheo bronchial lymph node) • vasoconstriction and peri-arteritis, eosinophilic, acute, marked (mesenteric artery) • skin lesions, multifocal, acute, moderate • pulmonary oedema focal acute mild • bronchitis multifocal acute to subacute mild associated with parasitical infestation • oral ulci multifocal acute mild • cholangitis, fibrosing, chronic, diffuse, mild. • gastritis, eosinophilic, superficial, subacute, mild. (pyloric stomach) • granulomatous gastritis focal chronic mild associated with parasites (pyloric stomach) • adrenocortical necrosis, multifocal, peracute, moderate. • venous thrombosis, local, chronic (colonic serosal vein) • Thyroid necrosis, diffuse, peracute, marked	• Bronchopneumonia caused by bacterial infection of the lungs • bronchopneumonia necrotizing, suppurative, diffuse, acute, moderate, associated with bacterial infection (mixed coliforms).		This male juvenile harbour porpoise in moderate body condition died during transport to the rehabilitation centre after having been trapped in a gillnet for over 30 minutes. The proximal cause of death is likely the bronchopneumonia caused by bacterial infection of the lungs. The most likely source of these bacteria is aspirated contents of the digestive tract. No signs of aspiration of seawater were found. Histological examination of the thyroid gland gave no indication of malfunctioning. The question why this animal was so small remains unanswered.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP080403	• Lung: nematode infection of the alveoli, mild. • Pulmonary blood vessel: nematode infection, mild. • Lymphadenitis (pulmonary) multifocal moderate acute • Proximal intestine: enteritis, eosinophilic, focal, subacute, mild.	• Dermal trauma (blowhole) multifocal acute severe • Lung (left cranioventral lobe): bronchopneumonia, suppurative, locally extensive, acute, marked.	• Pulmonary oedema diffuse moderate acute	This juvenile female harbour porpoise was found alive in moderate nutritional condition. Severe trauma was inflicted to the blowhole and underneath the right eye and involving the eyelids of both eyes. Due to the severity of the trauma whereby the animal was unable to close the blowhole, the harbour porpoise was killed with a toxic injection. The most significant diagnosis was an acute focal pneumonia and a mild infection of the pulmonary blood vessels. However, these diagnoses seem unlikely to have caused the emaciation of the harbour porpoise. By default, it must be concluded that the head injury made the animal unfit to feed and hence its nutritional condition declined.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP080701	• Lymphadenitis multifocal haemorrhagic acute moderate • Splenitis diffuse acute moderate • Liver: bile duct hyperplasia, diffuse, marked. • Sero hemo pericard diffuse acute mild • Pancreatic duct hyperplasia, focal, moderate. • Adrenocortical haemorrhage and necrosis, multifocal, acute. • Pneumonia multifocal chronic mild associated with nematodes • Hepatitis focal chronic (to healed) mild	• Interstitial pneumonia, haemorrhagic, diffuse, acute, marked, associated with Staphylococcus aureus infection. • Pleuritis bilateral focally extensive fibrinopurulent acute moderate. • Pneumonia, lymphohistiocytic, locally extensive, chronic, marked. • Pancreatic angitis, suppurative, necrotizing, chronic-active, multifocal, marked.	• Sepsis • Pulmonary oedema diffuses acute moderate	This juvenile female harbour porpoise died of an acute haemorrhagic pleuro pneumonia which turned into a sepsis. The hepatitis was chronic. The aetiology is unclear as is the significance of this hepatitis to the health of the animal. Likewise, is the aetiology and significance of the sero hemo pericard unclear. The location of the infection in the medial rostral thirds of the lung indicate a bacterial infection introduced by inhaling the infectious agent. The fairly marked infestation of the pulmonary blood vessels with nematodes has an unclear significance. Together, this porpoise had a chronic problem of liver and pancreas (cause remains to be determined: check blood for bile acids) and an acute problem of the lung: S. aureus infection with spread to other organs. In addition, there was a more chronic problem of the lung, which was negative for mycobacteria by ZN stain or other pathogens by Grocott and Gram stains.
PP081222	• Epiglottis: epiglottal ulcer, focal, acute, mild. • Teeth: Attrition, generalized, marked. • Pharynx: pharyngitis, necrotizing, multifocal, acute, mild. • Oesophagus: oesophagitis, necrotizing/ulcerative, multifocal, acute, mild. • Forestomach: parasite infection. • Bile ducts: cholangitis, fibrosing, diffuse, chronic, moderate. • Heart and pulmonary trunk: parasite infection. • Skin: ulceration and scars, multifocal, chronic, mild.	• Lungs: bronchopneumonia, suppurative, multifocal or coalescing, chronic-active, marked, associated with parasite infection (and bacterial infection?) • General: emaciation, marked.		The most significant lesion was the bronchopneumonia, likely with both a parasitic and bacterial component.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP090626	• Tracheo-bronchitis, lymphoplasmacytic, diffuse, superficial, chronic, mild. • Bronchopneumonia, histiocytic, diffuse, chronic, mild, associated with nematode infection. • Metritis, lymphocytic, diffuse, superficial, chronic, mild. • Lung associated lymph node, intestinal lymph node: lymphadenitis, eosinophilic, diffuse, acute, mild. • Cholangitis multifocal chronic mild associated with parasitic infection • Galactophoritis, lymphoplasmacytic, superficial, chronic, mild. • Pulmonary arteriitis multifocal mild associated with parasitic infection • Dermatitis multifocal acute mild	• Hepatic lipidosis, moderate diffuse. • Hepatitis, necrotizing, suppurative, focal, acute, mild. • Bronchiolitis obliterans, multifocal, subacute, moderate		This adult female in good nutritional condition died within hours of being found on the beach. The cause of death is unknown. Brucella ceti was cultured form lung tissue, pulmonary lymph node and spleen. Possibly the cow had aborted previously although the time of stranding coincides with the normal calving season. Taking all results together, the most significant lesion is the hepatic lipidosis, which is associated in some areas (but too little liver tissue examined histologically until now) with hepatocyte necrosis and acute inflammation. This is reminiscent of acute ketosis of lactating cows, or pregnancy toxaemia of sheep. However, the pathologic changes in the liver are not convincing and more liver samples from different parts of the liver need to be examined histologically. Another lesion that might be significant is the bronchiolitis. In about 2/3 of the observed bronchioles in the left and right lung, the lumen is obstructed either by the presence of inflammatory cells or by contraction of the smooth muscle. It is not clear how much effect this would have on respiratory function, but it is likely that it would decrease it. The cause of the bronchiolitis is most likely from nematode infection. These were found at one location in the alveolar lumina, although none were observed in the bronchioles in the tissues examined microscopically.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP100105	• Oesophageal ulcers multifocal acute mild • Corneal ulcer focal chronic mild • Pyloric stomach: gastritis focal chronic mild associated with a fungal (yeast) infection and the presence of nematodes • Middle ear: otitis diffuse chronic mild associated with the presence of nematodes • Hepatic lipidosis diffuse acute mild • Oesophagus, fundic and pyloric stomachs: yeast-like infection. • Dermal lesions multifocal acute mild likely due to trauma related to stranding • Dermatitis multifocal chronic mild • Lung-associated lymph node: benign lymphoid hyperplasia, moderate. • Pulmonary blood vessel: nematode infection. • Liver: hepatic lipidosis, diffuse, mild. • Intestine: enteritis, ulcerative, focal, acute, mild. • Skeletal muscle: muscle degeneration, multifocal, acute, moderate. • Skeletal muscle: muscle atrophy, diffuse, chronic, moderate. • Pancreas: ductular hyperplasia, chronic, moderate		• bronchopneumonia suppurative, granulomatous, diffuse, chronic-active, associated with mixed bacterial and yeast-like (?) infection and aspiration of food remains marked.	This juvenile male harbour porpoise with a very poor nutritional condition was euthanized after 48 hours in the rehabilitation centre. The main lesions found were broncho-pneumonia besides emaciation. The respiratory organs showed three different types of lesions. First small calcified nodules which will not have had any significance (probably lungworms). Second major areas of lung which were consolidated and third large parts of lung which had a different colour and was slightly firmer (both likely aspiration pneumonia). The wasting of the back muscles in the presence of a relatively thick blubber layer is likely due to the animal preferentially fulfilling its calorie needs form muscle digestion rather than to threaten its insulation by digesting its isolating layer of blubber. The significance of the pancreatic duct hyperplasia is unclear. The proximal cause of death (or reason to euthanise) is the aspiration bronchopneumonia. The ultimate reason which will have caused the emaciation is unclear, possibly a mycotic infection of the gastro intestinal tract or maldigestion due to pancreatic malfunction.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP100928.1	• Dermal incision wounds multifocal acute mild • Pulmonary hypostatic congestion diffuse mild		• Pulmonary oedema, diffuse, acute, moderate.	This male neonatal harbour porpoise in poor level of nutrition was euthanized as no artificial milk formulas are known that can maintain neonatal harbour porpoises. The poor level of nutrition and empty intestines indicate the animal had not been feeding recently. The observed stomach ulcers may have been caused by the associated stress and malnutrition. The incision wounds in the skin may have occurred due to contact with the ground during the stranding process.
PP100928.2	• • Oesophagus: oesophageal ulceration, multifocal, acute, mild. • • Fundic stomach lesions unknown aetiology		• • Lung: pulmonary oedema, diffuse, acute, moderate. (or artefact)	This female neonatal harbour porpoise in poor body condition was euthanized as no artificial milk formulas are known that can maintain neonatal harbour porpoises. The longitudinal ulcers in the oesophagus may have been caused by damage during tube feeding. The lesions in the fundic stomach are of unknown aetiology and significance.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP110228	• Renal tubular epithelial degeneration, multifocal, acute, mild, associated with protein casts (myoglobinuria). • Alveolitis, suppurative, diffuse, acute, mild, associated with microthrombi. • Tracheo-bronchial lymph node: lymphoid hyperplasia, moderate. • Hepatic congestion diffuse chronic mild • Renal pelvic haemorrhage focal acute mild • Pulmonary nematode infection, very mild. • Hepatic lipidosis, diffuse, mild. • Splenic lymphoid hyperplasia, mild. • Pyloric stomach: gastric haemorrhage, focal, acute, mild	• Oesophagitis ulcerative multifocal to confluescent acute marked	• Pulmonary oedema diffuse peracute severe	This juvenile male harbour porpoise in moderate nutritional condition was euthanized after 5 days in the rehabilitation centre. The most important diagnosis was severe ulcerative inflammation of the oesophagus and a severe gastritis in the pyloric stomach. The porpoise had a fatty liver which may indicate an abnormal metabolism of fatty acids possibly caused by "partial" fasting. The importance of the pulmonary lesion is unclear, lung associated lymph nodes were enlarged but possibly these lymph nodes drain the oesophagus as well (unknown to me for this species). Based on the epithelial damage of the digestive tract at multiple site a viral aetiology has to be considered.
PP110329	• Oesophagus: oesophagitis, granulomatous, focal, chronic, mild, associated with migrating parasite. • Fundic stomach, pyloric stomach: vasculitis, granulomatous, multifocal, chronic, moderate, associated with nematode infection • Pyloric stomach: Focal gastritis, ulcerative, granulomatous, locally extensive, chronic, marked, associated with parasite infection (Pholeter gastrophilus?). • Dermatitis, erosive, suppurative, chronic-active, moderate, associated with bacteria and yeast-like organisms • Lung-associated lymph node, spleen, prescapular lymph node: lymphoid hyperplasia, benign, moderate. • Diffuse adrenocortical hyperplasia chronic severe 3) Incidental diagnoses: • Pulmonary artery: arteritis, lymphocytic, diffuse, chronic, moderate, associated with nematode infection.	• Bronchopneumonia multifocal, chronic, moderate, associated with nematode infection. • Bronchopneumonia multifocal, acute, marked, likely associated with bacterial infection. • Focal ulcerative chronic gastritis severe (fore stoma		This adult female harbour porpoise in very poor nutritional body condition died during transport with symptoms of respiratory distress. Probably the most significant lesion is the mixed bacterial and parasitic bronchopneumonia. It is not clear whether the bacterium Actinobacillus delphinicola contributed to this inflammation, since the association between this newly discovered bacterium (in 1996) and disease is not known. The second most important lesion is a very large stomach ulcer in the fore stomach.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP110711	• Splenomegaly diffuse acute mild (lymphoid hyperplasia, mild) • Pulmonary arterial parasitic infection multifocal chronic moderate • Bronchio-tracheal parasitic infection multifocal chronic moderate • Middle ear parasitic infection diffuse chronic moderate • Dermal trauma multifocal acute to subacute mild • Oesophageal ulceration multifocal chronic mild	• pneumonia, pyogranulomatous, locally extensive, chronic, marked. • Emaciation diffuse chronic severe • Lymphadenitis multifocal subacute to chronic marked (Tracheo-bronchial lymph node, prescapular lymph node: lymphoid hyperplasia, marked.) • Hepatocytic degeneration, random, multifocal, acute, moderate, with neutrophil infiltration and hepatocytic necrosis	• Pulmonary oedema acute severe	This juvenile female harbour porpoise in poor nutritional condition died after being admitted less than 24 hours in the rehabilitation centre. The main pathological findings were a very poor nutritional condition, a chronic pneumonia, in combination with splenomegaly, multiple hyperplastic lymph nodes and acute hepatocytic degeneration. It might be speculated that the animal had been unable to forage effectively, possibly due to the effects of the focal pneumonia, which caused a decrease in immuno-resistance, which resulted in the local pneumonia turning into sepsis with hepatocytic degeneration due to toxin release or bacterial leakage from the intestines. Brucella ceti is the most likely etiologic agent. Aspergillus was cultured from a lung sample but not found on histology. It is not clear how this juvenile animal (1 or 2 years old) contracted a Brucella ceti infection

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP110928.1	• Dermal trauma focal acute mild • Corpus alienum (sand) in the air-sacs multifocal acute moderate • Fundic stomach: gastric erosion, focal, superficial, acute, mild	• Kidney: renal tubular epithelial cell vacuolation, diffuse, marked. • Liver: hepatocytic vacuolation, diffuse, marked.	• Pulmonary atelectasis multifocal acute (or possibly post mortal due to resorption) • Lung: pulmonary oedema, diffuse, acute, moderate.	This neonatal female harbour porpoise in moderate level of nutrition was euthanized after 2 days in rehabilitation. The main finding on necropsy were vacuolization of hepatocytes and tubular epithelium of the kidneys. These lesions are in accordance with the signs noted which were strong defence musculaire on admission, electrolyte imbalance and the continuous vomiting of administered fish gruel. The tubular epithelial vacuolation in the kidneys and hepatocytic vacuolation in the liver resembles a lysosomal storage disease, like beta-mannosidosis in cattle, or an intoxication, like Swainsona intoxication. Glycogen storage disease type 1 (von Gierke disease) specifically affects liver and kidney (Vet Pathol. 1995 Sep;32(5):460-5.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP110928.2	• Lung: alveolitis, pyogranulomatous, focal, chronic, mild, associated with nematode infection. • Pulmonary artery: nematode infection, mild. • Bronchus: nematode infection, moderate. • Pulmonary-associated lymph node: benign lymphoid hyperplasia, mild. • Liver: bile duct hyperplasia, focal, chronic, marked.	• Dermal trauma acute multifocal to coalescing marked	• pulmonary oedema, diffuse, acute, marked.	This male juvenile harbour porpoise in good nutritional condition died during transport to the rehab centre. The proximate cause of death was the pulmonary oedema, which may have been caused by psychogenic shock due to stress and pain. The animal was severely wounded by predators. It is unclear whether this happened at sea (e.g. by grey seals) which would then cause stranding or post stranding by birds and terrestrial scavengers (foxes and dogs). The observations on the respiratory tract do not lead to clear conclusions on the pathology observed. There could either be an insignificant old focal pneumonia or possibly an acute pneumonia which may have been a reason for the animal to strand. Histology will give further clues. The observed bite wound on the right tail fluke is interesting. This bite wound may well be the result of an attack by another cetacean.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP111219	• Dermal lesion chronic mild • Lung: nematode infection of pulmonary vein, mild. • Tracheo-bronchial lymph node: benign lymphoid hyperplasia, mild. • Pancreas: pancreatic collecting duct hyperplasia, diffuse, chronic, mild.	• Bronchopneumonia, haemorrhagic, suppurative, diffuse, acute, marked, associated with bacterial infection • Lung: alveolitis, pyogranulomatous or granulomatous, multifocal, chronic, mild or marked, associated with nematode infection.		This juvenile male harbour porpoise in moderate to poor nutritional condition died after five days in the rehabilitation centre. The main finding at necropsy was the pneumonia which had a chronic component strongly associated with a nematode infection and an acute component which was purulent and not associated with a nematode infection. The acute bacterial bronchopneumonia was responsible for the death of the animal. Location (ventral parts of the lung), the observation of several different species of bacteria and the history with tubing and frequent vomiting suggest the possibility of an aspiration pneumonia, although no food remains were seen on histology.
PP120703	• Dermal and subdermal trauma multifocal acute moderate • Gastritis multifocal acute to subacute mild. • Lung: bronchiolitis, suppurative, diffuse, acute, mild. • Eyelid: conjunctivitis, suppurative, superficial, acute, mild.		• Pulmonary oedema diffuse acute moderate.	This neonatal harbour porpoise in moderate nutritional condition was euthanized. The main findings were an absence of milk or nutritional content in the stomach and bowels and presence of sand throughout the digestive tract. The presence of sand throughout the digestive tract and the presence of multiple small erosions in the fore stomach indicate this animal had been ingesting sand previous to its stranding. Lack of nutrition and consequent hunger may have caused this animal to ingest sand; possibly separation of its mother is the ultimate cause of the animal's stranding.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP120906.1	• Lung: nematode infection in alveoli. • Bronchitis, multifocal, lymphocytic, chronic, associated with nematode infection. • Verminous arteritis multifocal acute to chronic mild (pulmonary arteries) • 3) Incidental diagnoses: • Dermal trauma multifocal acute to subacute moderate • Stomatitis, ulcerative, focal, acute, mild. • Oesophageal ulcers multifocal acute to chronic mild • Benign lymphoid hyperplasia moderate • Verminous otitis diffuse chronic moderate • Pyloric stomach: granuloma, eosinophilic, focal, chronic, mild.	• Pneumonia, pyogranulomatous, multifocal to coalescing, chronic, marked, associated with fungal (Aspergillus fumigatus) infection. • Encephalitis, pyogranulomatous, multifocal, chronic, moderate, associated with fungal (Aspergillus fumigatus) infection. • Pulmonary haemorrhage and oedema diffuse acute marked		This male juvenile harbour porpoise in moderate nutritional condition died within one day at the rehabilitation centre. The most severe lesion by far is the pyogranulomatous pneumonia associated with Aspergillus fumigatus infection. Interestingly, the fungus has spread to the cerebellum, but is not present in sections of any other tissues. The pneumonia was severe enough to be responsible for the death of the animal.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP120906.2	• Lung: foreign body (trematode egg). • Lung-associated lymph node: venous nematode infection. • Subcutaneous emphysema diffuse acute moderate (possible artefact) • Dermatitis multifocal acute and chronic and healed moderate • Benign lymphoid hyperplasia multifocal acute to chronic moderate • Cholangitis, hyperplastic, multifocal, chronic, moderate, associated with trematode (Campula oblonga) infection. • Cutaneous papilloma's, multifocal, chronic, moderate. • Verminous otitis media diffuse chronic mild • Pyloric stomach: gastritis, fibrosing, multifocal, chronic, mild, associated with trematode (Pholeter gastrophilus) infection. • Renal calcification multifocal chronic mild • Lingual ulceration focal acute mild	• sepsis • Pneumonia, pyogranulomatous, multifocal, chronic, moderate, associated with nematode infection. • Interstitial pneumonia, suppurative, diffuse, acute, moderate. • Joint capsule left jaw: arthritis, suppurative, diffuse, acute, marked, associated with coccoid bacteria. • Hepatic abscesses, multiple, marked.	• Pulmonary oedema acute diffuse marked	COMMENTS: This juvenile female harbour porpoise in moderate to poor nutritional condition died spontaneously during transport. The suppurative arthritis and the coccoid bacteria arranged in strings within the exudate fits with Streptococcus dysgalactiae infection. It is difficult to determine where this S. dysgalactiae infection started: mandibular joint, lung (secondary to lungworm infection), or liver (secondary to liver fluke infection). The diffuse presence of neutrophils in the alveolar walls (suppurative interstitial pneumonia) suggests that the bacterium has spread to the blood (sepsis), which would be the most likely proximate cause of death. Interestingly, the mammary gland had no evidence of S. dysgalactiae infection, even though this bacterium is a well-known cause of mastitis in cattle. The arthritis of the mandible may well have hindered the animal in hunting and feeding and be as such a more ultimate cause of death.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP120906.3	• Adrenalitis, necrotizing, multifocal, chronic, with eosinophilic intranuclear inclusions. • Hemoperitoneum acute diffuse moderate (if not artefact) 3) Incidental diagnoses: • Cholangitis, lymphocytic, diffuse, chronic, marked, with marked periductular fibrosis, associated with trematode (Campula oblonga) infection. • Lip: cutaneous ulcer, superficial, focal, acute, mild. • Blowhole ulcer: epidermal necrosis, focal, acute, mild, associated with eosinophilic intranuclear inclusions. • Pyloric stomach: gastritis, granulomatous, focal, chronic, mild. • Skin fin: incision wound, superficial, chronic, mild.	• Bronchopneumonia, granulomatous, multifocal, chronic, moderate, associated with nematode infection. • Interstitial pneumonia, suppurative, histiocytic, locally extensive, chronic, moderate. • Cerebrum: polioencephalitis, multifocal, mild. • Hepatitis, necrotizing, multifocal, acute, marked.	• Pulmonary oedema acute diffuse marked	This male juvenile harbour porpoise in moderate nutritional condition was euthanized after 8 days in the rehabilitation centre. Clinical symptoms indicate a peracute severe infection, possibly sepsis (hemoperitoneum) pneumonia (focal pneumonia) or an infection of the CNS (no gross pathological indications). Based on the presence of eosinophilic intranuclear inclusions in the adrenal gland and in the blowhole ulcer, there is evidence of a systemic herpesvirus infection. This would fit with the neuronophagia suspected in the cerebrum, as well as the necrotizing hepatitis. These pathological changes fit with the aberrant behaviour observed in this animal during rehabilitation.
PP121031	• Bronchitis, lymphoplasmacytic, diffuse, chronic, mild. • Verminous arteriitis multifocal acute to chronic moderate • Benign lymphoid hyperplasia multifocal acute moderate • Acetonemia • Cholangitis, fibrosing, hyperplastic, diffuse, chronic, moderate, associated with Campula oblonga infection. • Renal cyst. • Thymic cysts. • Aorta: atheroma (?), mild. • Mesenteric lymph node: lymphadenitis, eosinophilic, focal, acute, mild. • Prescapular lymph node: benign lymphoid hyperplasia. • Fore stomach: scar, superficial, focal. • Epidermal and dermal scars, multiple, mild	• Bronchopneumonia, pyogranulomatous, necrotizing, multifocal, chronic, moderate, with suspect eosinophilic intranuclear inclusions, associated with Salmonella sp. infection and nematode infection.	• Pulmonary oedema, diffuse, acute, moderate	This gravid adult female in moderate body nutritional condition died shortly after starting the transport to the rehabilitation centre. The main lesions were a diffuse acute bronchopneumonia associated with Salmonella sp. and a moderate to severe infestation of the pulmonary arteries with nematodes. The eosinophilic intranuclear inclusions in the epithelial cells of the bronchus are suspect of herpesvirus infection. The acetonemia, empty stomach and slightly wasted musculature indicate she had not been feeding for quite a while. Likely she did not have the necessary fitness to hunt caused by either a lack of oxygen due to the pneumonia and ill feeling caused by the toxins of infection or by the disturbed circulation due to the infestation of the pulmonary arteries with nematodes. Her proximate cause of death is unclear.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP121102	• Bronchopneumonia, lympho-plasmacytic, diffuse, chronic, associated with nematode infection. • Lung-associated lymph node, prescapular lymph node: benign lymphoid hyperplasia • Dermal scars multifocal chronic mild • Dermal wound multifocal acute mild • Subdermal scar focal chronic mild • Cholangitis, fibrosing, diffuse, marked, associated with Campula oblonga infection. • Ascites very mild (normal?) • Hydropericardium very mild • Hydrothorax very mild	• Pneumonia, granulomatous, coalescing, chronic, marked, associated (in some foci) with fungal structures.		This adult male harbour porpoise in good to moderate nutritional condition died after three days in rehabilitation. The main finding was a severe pneumonia associated with Aspergillus fumigatus and associated hyperplasia or inflammation of the draining lymph nodes. Scars on the tail stock indicate this animal was once caught in a line which tightened around the end of its tail stock. An associated hard swelling might indicate bony involvement. The proximate cause of death is most likely the pneumonia and associated phenomena. (toxins, laboured breathing, hypoxia).

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP121130	• Pulmonary hyperaemia acute to chronic multifocal mild and associated with pneumonia • Pulmonary arteritis multifocal chronic mild associated with verminous infection. • Benign lymphoid hyperplasia multifocal chronic moderate • 3) Incidental diagnoses: • Pulmonary oedema peracute diffuse mild • Oesophageal ulceration multifocal chronic mild • Hydropericardium diffuse mild • Dermal trauma multifocal chronic mild possible rake or bite marks(?).	• Asphyxia due to pneumonia in combination with hydrops ascites. • Bronchopneumonia pyogranulomatous, locally extensive, chronic, moderate, associated with large adult nematodes (Torynurus convolutus, Halocercus invaginatus) and small nematode larvae (Stenurus minor). • Forestomach, jejunum, colon: vasculitis, leukoclastic, haemorrhagic, multifocal, chronic-active, marked • Ascites diffuse severe. • Uterus: vasculitis, leukocytoclastic, haemorrhagic, multifocal, subacute, mild. • Dermatitis, pyogranulomatous, multifocal, chronic, marked, with epidermal hyperplasia, keratin pearls, and bacterial infection.		This juvenile female harbour porpoise was emaciated and died after 17 days in the rehabilitation centre. Unusual lesions in this porpoise are those of leukocytoclastic vasculitis of the small and medium-sized arteries of the stomach, intestine, and (to a much milder degree), uterus. I have never seen vascular lesions like this in harbour porpoises. Since the lesions extended to the lumen of the intestine, they would have resulted in blood loss into the intestinal lumen, and thus fit with the anaemia diagnosed clinically. Since the lesions also extended to the serosal surface, they also fit with the mild hydrops ascites observed at gross autopsy. The protrusion of the teats and clitoris and vulvar mucosa from the genital slit were caused by pressure from the ascites. The combination of anaemia, pressure on the thorax form the distended abdomen and the pulmonary infection, including the verminous infection of the pulmonary arteries are likely to have compromised the breathing, circulation and oxygen supply of the animal and this is probably the proximate cause of its death. The skin lesions, with apparent invagination of the epidermis, resembles an inverted papilloma. There appears to be an associated increase in vascularization of the subjacent dermis. It seems to fit well with papillomavirus infection, and needs to be followed up. The bacterial infection and subsequent inflammation seem to be secondary.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP130204	• Dermal lesions multifocal chronic mild • Dermal lesions multifocal acute mild • Lingual ulcer focal acute mild • Liver: bile duct hyperplasia, diffuse, chronic, mild. • Brain: medulla oblongata, malformation. • Urinary bladder: serosal haemorrhage, multifocal, acute, mild. • Pyloric stomach: gastric ulceration, focal, acute, mild, with subjacent haemorrhage. • Oesophagus: oesophageal ulceration, multifocal, acute, mild. • Corneal perforation acute focal marked	• Skeletal muscle: muscle degeneration, locally extensive, acute to subacute, marked. • Kidney: urolithiasis, mild. • Right heart: myocardial oedema, multifocal, acute, mild.	• Pulmonary oedema, diffuse, acute, mild.	This juvenile male harbour porpoise in in moderate level of nutrition and fresh state of autolysis as humanely killed as there was an inability to administer sufficient calories and the animal continued to lose weight. Significant findings were subperitoneal haemorrhages on and close to the urinary bladder and pale muscles suggesting necrosis. Histology confirmed pathology of the muscles. However, the muscles were not necrotic. Blood values indicated kidney failure (increasing urea up to 30(20 is high normal) and creatinine from 19 to 80 and Na from 144 to 165), which probably caused the vomiting and may also have caused protein loss and wasting despite the food intake. The proteinaceous material observed in the distal renal tubuli may therefore be more significant then they appeared. Speculatively the possibility of an attack or blunt trauma, causing subserosal haemorrhages on the bladder) followed a strong flight reaction and capture myopathy might be considered. It is worthwhile to see if cardio respiratory clinical data can be evaluated to see if any signs of heart muscle pathology were observed.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP130513	• Skin: parakeratotic hyperkeratosis, locally extensive, marked, associated with bacterial and fungal (Fusarium sp.) infection. • Icterus diffuse acute mild • Subpleural hematoma focal chronic mild • Esophagitis ulcerative multifocal chronic mild • Stomach, pyloric: gastric abscess, focal, chronic, mild, associated with a trematode infection (probably Pholeter gastrophilus) • Cardiac valve cyst focal chronic mild • Keratitis bilateral chronic moderate • Adrenal gland: Hypoplasia of the inner layer of the adrenal cortex, diffuse, chronic, marked, and replacement by erythrocyte-filled blood vessels. • Cholangitis, multifocal, chronic, mild, associated with trematode infection • Thyroid gland: cyst, solitary.		• Pulmonary oedema diffuse acute severe	This adult female in good nutritional condition was euthanized after six months in rehabilitation as she did not dive and only floated at the surface. The cause of this problem was not determined during necropsy. The most significant problem was a multifocal dermatitis caused by a Fusarium sp infection. In humans such an infection does occasionally disseminate and result in arthritis and osteomyelitis. Although such lesions were not observed they could have been missed during necropsy. The mild icterus and hepatic swelling may have resulted from the therapy with voriconazole or the high dosages of prednisolone which were administered. The keratitis may also have been caused by a Fusarium sp infection. The hypoplasia of the inner layer of the adrenal cortex could have been caused by a neoplasm elsewhere in the body producing sex steroids, as this would result in the zona reticularis to become hypoplastic. The zona reticularis is responsible for producing sex steroids. Alternatively, it might be a consequence of aging: teleangiectasis of the adrenal cortex, at the cortico-medullary border, with loss of adrenocortical cells, occurs in older animals. A third possibility is that it is a consequence of high prednisolone doses 1 to 2 months before euthanasia.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP130829	• Lung-associated lymph node, right: lymphoid hyperplasia, benign, marked. • Liver: Cholangitis, proliferative, multifocal, chronic, marked, with fibrosis. • Dermatitis, lymphocytic, diffuse, chronic, mild. • Otitis diffuse chronic mild associated with Stenurus minor infection • Bronchitis multifocal chronic mild associated with nematode infection • Stomach, fundic: gastritis, lymphocytic, focal, chronic, mild, associated with parasite infection. • Duodenal ampullae: enteritis, lymphocytic, multifocal, chronic, associated with parasite infection. • Mesenteric lymph node: lymphadenitis, lymphocytic, focal, chronic, mild, associated with parasite infection. • Oesophagitis ulcerative multifocal subacute moderate	• Lung: pulmonary abscesses, multifocal, chronic, marked, associated with Aspergillus fumigatus infection. • Lung: bronchopneumonia, pyogranulomatous, coalescing, chronic, marked, probably associated with Aspergillus fumigatus infection. • Palate: palatal abscess, focal, chronic, marked, associated with Aspergillus fumigatus infection.		This adult male in very poor nutritional condition died due to a chronic pneumonia. The most important lesions are those in the palate and lungs, caused by Aspergillus fumigatus infection. These were chronic lesions that would explain the emaciation of this animal. Dissemination from one location to the other by hematogenous spread or via the airways appears likely. It is surprising that breathing was normal upon acceptance into the rehabilitation centre, because he must already have had severe respiratory lesions then. Aspergillus fumigatus infection usually is secondary to some kind of immunosuppression. It is not clear what that was in this animal.
PP140204	• Cerebellum: Purkinje cell degeneration, multifocal, acute, moderate (or artefact from, e.g., euthanasia?) • Liver: hepatic lipidosis, diffuse, marked.		• Lung: pulmonary oedema, diffuse, peracute, moderate. (associated with euthanasia)	This neonatal harbour porpoise in poor nutritional condition was euthanized as a consequence of its poor survival chances. The most important lesion was the poor level of nutrition and complete absence of food remains in the gastrointestinal tract which indicate starvation was the main problem of this neonate.

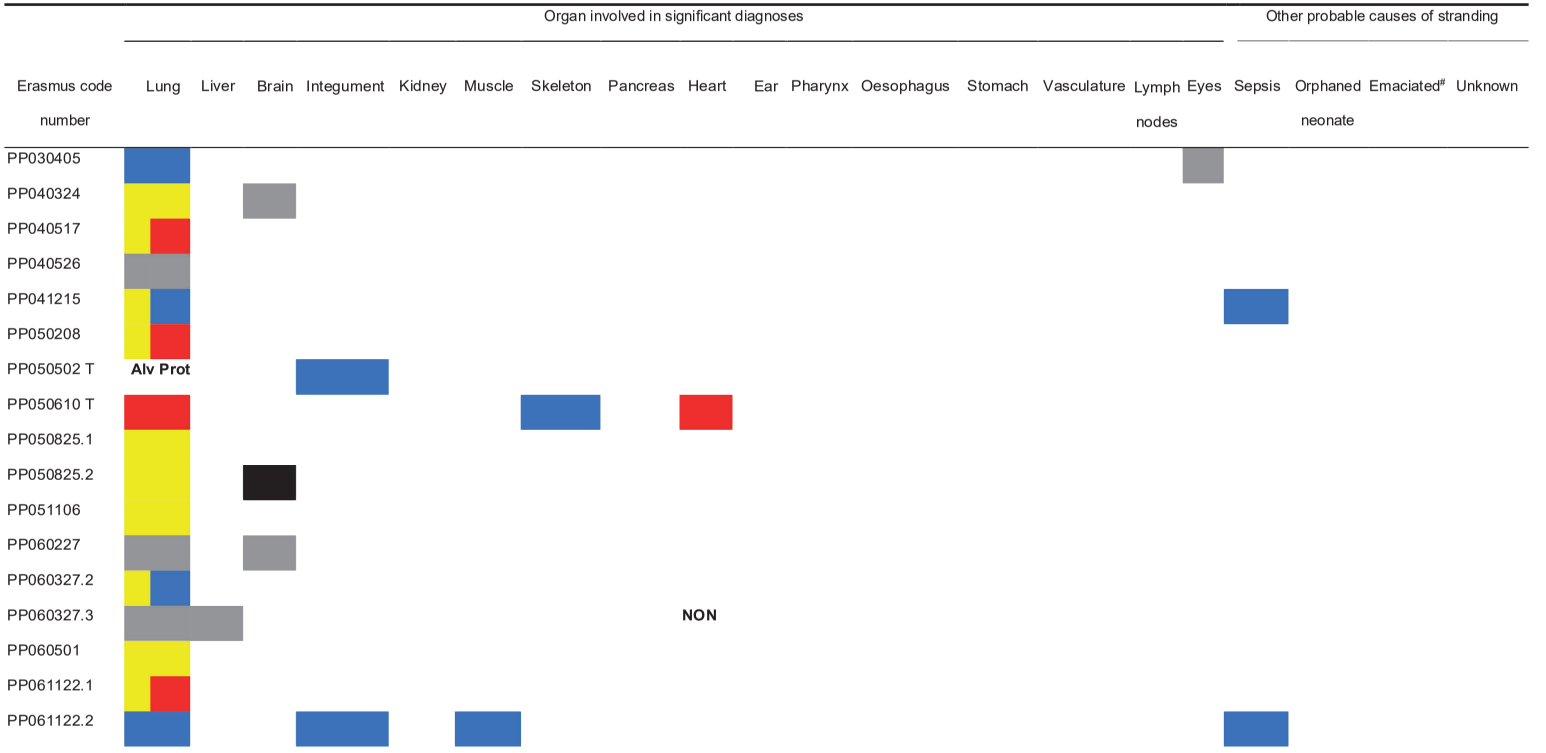
Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP140627	• Epidermal lesions multifocal acute mild • Ulcerative esophagitis multifocal acute mild • Ulcerative gastritis multifocal acute mild • Lung: bronchopneumonia, locally extensive, fibrinosuppurative, mild, acute. • Liver: hepatic lipidosis, diffuse, moderate. • Umbilicus: omphalitis, haemorrhagic, suppurative, acute, focal, mild. • Tonsil: tonsillitis, suppurative, focal, superficial, acute, mild.		• Pulmonary oedema diffuse peracute marked (caused by euthanasia)	This female neonate harbour porpoise in moderate nutritional condition was euthanized upon arrival in the rehabilitation centre of SOS-Dolfijn. The most significant observations were absence of food remains or chillum in the mesenteric lymphatic vessels and the liver degeneration. The most likely cause of death in line with these observations is starvation. The liver degeneration may have caused the anorexia or might be the result of the anorexia. The multiple small ulcers observed in the gastrointestinal tract might have been the result of the chronic stress due to not feeding. These ulcers appear too small to have caused the anorexia. The inability to feed may have been the result of the mother animal not being available to the neonate.
PP140917	• Oedematous lymphadenitis multifocal acute moderate (prescapular Lnn) • Lymphopathy multifocal acute moderate (pulmonary Lnn) • Lung: pulmonary oedema, diffuse, acute, mild to moderate. • Hepatic lipidosis diffuse mild • Prescapular lymph node: Lymphonodular oedema, diffuse, acute, marked. • Lung-associated lymph node: Lymphoid hyperplasia, benign, marked.	• Lung: bronchopneumonia, suppurative, multifocal, acute to subacute, moderate to marked, associated with bacterial infection. • Malnutrition (no nourishment) chronic • Skin of snout: Dermatitis, suppurative, necrotizing, locally extensive, superficial, acute, marked, associated with bacterial infection		This neonatal male harbour porpoise in poor nutritional condition was euthanized as the length indicated this animal was unfit for release. The most significant findings were a bronchopneumonia and a marked locally extensive dermatitis of the snout. The oedematous lymphadenitis of the prescapular lymph nodes and the enlarged pulmonary lymph nodes were consequential to the above-mentioned infections.

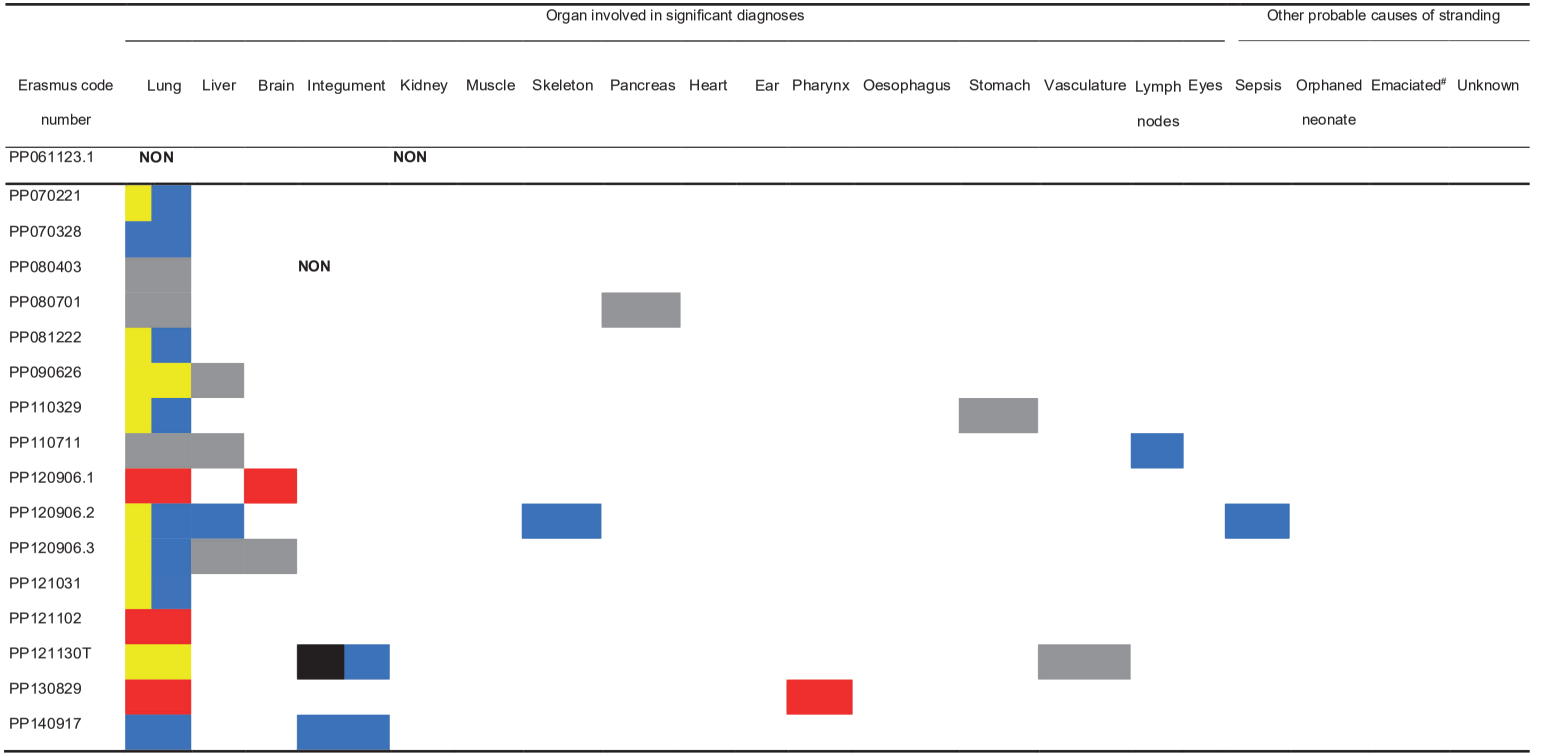
Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP150210	• Ventricular septal defect, congenital • Pneumonia, granulomatous, multifocal, chronic, mild. • Pulmonary oedema, locally extensive, acute, moderate. • Lung-associated lymph node: lymphoid hyperplasia, benign. • Ascites acute diffuse mild • Thyroid hypoplasia or thyroid follicular atrophy, marked. • Dermal trauma acute multifocal mild • Dermal hyperplasia focal chronic mild • Duodenum: enteritis, granulomatous, multifocal, chronic, mild. • Mesenteric lymph node: lymphadenitis, granulomatous, eosinophilic, multifocal, chronic, mild • Epidermal keratin plug, focal.			This female harbour porpoise in good nutritional condition was euthanized after 11 days in the rehabilitation centre. The most significant lesion was a ventricular septal defect. It is unclear if the observed pulmonary oedema was due to heart failure or to euthanasia. During life no signs were observed which indicate heart failure, no fluid in thorax or abdomen, normal to low breathing frequency. The pulmonary associated lymph nodes were enlarged, indicating possibly a pneumonia which could otherwise not be supported by any other observations.
PP151116	• Pericardial effusion mild • Persistent ductus botallicus chronic mild • Mesenteric lymph node: Lymphonodular necrosis, multifocal, acute, moderate.	• Tail vertebra: Puncture wound with suppurative osteitis, focal, acute, superficial, mild. • Skin: Puncture wounds with suppurative dermatitis, multifocal, acute to subacute, superficial, mild • Emaciation severe • Lung: Bronchopneumonia, fibrinopurulent, unilateral, locally extensive, acute, moderate.	• Pulmonary oedema peracute diffuse severe associated with euthanasia or agony	This juvenile female porpoise in extremely poor nutritional condition was euthanized because of poor prospects for recovery. The main findings were emaciation and multiple trauma suspected to be caused by a grey seal attack. The retracted wound edges and bulging tissue indicate this trauma was caused approximately one to a few days before the animal stranded. Tens of small otoliths were observed in the fore stomach otherwise the digestive tract was near empty. Harbour porpoises of this length/age at this time of year still nurse and are mother dependent A possible sequence of events is then: loss of mother leading to loss of nutrition leading to emaciation and decrease of fitness and immune resistance with subsequent grey seal attack and bronchopneumonia. The bite wounds caused an osteomyelitis of the underlying vertebra.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP160304	• Lung, right cranial lung lobe: multifocal mild subacute granulomatous bronchopneumonia with intralesional nematodes • Lung, near left bronchus: focal extensive mild subacute pyogranulomatous bronchopneumonia with intralesional nematodes • Lung: left and right lung: multifocal mild subacute suppurative bronchopneumonia with intralesional bacteria • Skin, tailstock and fluke: multifocal moderate subacute necropurulent dermatitis, due to suspect bite marks • Liver: multifocal (random) moderate acute hepatocellular necrosis • Skeletal muscle: focal mild subacute skeletal muscle necrosis • 3) Incidental diagnoses: • Tail, vertebral column: mild scoliosis • Skin: focal mild chronic pyogranulomatous furunculosis • Skin: multifocal epidermo-pathy • Liver: multifocal mild chronic lymphoplasmacytic cholangitis	• General: Emaciation • Kidney: suspect protein loss		This severely emaciated juvenile female harbour porpoise showed very little autolytic changes. The lacerations and punctures in the skin of the tail fluke, pectoral fins and the tailstock were bilaterally present, often mirroring, with regular distances between them and very suggestive of (grey) seal bite wounds. The inflammation in the tissues adjacent to the wound seemed subacute (hyperaemia and suppurative exudate present, but little hyperplasia of epidermis or fibrosis) so could have been inflicted around the time of stranding. The irregular surface of the body wall seemed to be due to slight fluctuation of the thickness of the epidermis. The cause for this is not clear, but possibly related to drying of the skin due to the stranding. The skin on the right lateral body wall was different, and showed wrinkling in cranio-caudal direction, and might also be related to drying (on what side was the animal found?). A focal area of muscle necrosis was detected macroscopically and microscopically. Not the complete longissimus dorsi seemed to have been affected, it is not clear how much muscle was affected. The reported (clinical history) increased CK and LDH in the blood suggest that muscle damage was more widespread than the one focus detected. Within kidney cortex, both in glomerular tufts and in tubular lumina, was proteinaceous material. This could fit with the expected myoglobinuria, or with (more generalized) proteinuria (additional stains should be performed for definite diagnosis). The protein loss due to damage of the glomeruli (due to the myoglobinuria, if it occurred) might have caused the quick loss of weight and the hypoalbunemia. The cause for the liver necrosis and the skin lesions (epidermo-pathy) are not clear.

Erasmus code number	Lesions which did not contribute to stranding	Lesions which contributed to stranding	Lesions associated with agony (euthanasia) or acquired after stranding	Comment
PP160331	• Nematode infection of bronchioles • Lung artery: Nematode infection. • Focal dermal trauma healed (scar tissue) mild • Nodular hyperplasia of the gastric epithelium focal chronic mild		• Lung: Pulmonary oedema, locally extensive, acute, moderate.	This juvenile female harbour porpoise in good nutritional condition died shortly after she was found. Gross necropsy and histology did not provide clues to why this animal stranded. The only slightly unusual observation is that nearly all tissues had marked dilatation of small blood vessels, which were filled with erythrocytes. This suggests a widespread vasodilatation, which might cause hypovolemic shock (but what would cause such shock is unclear). In conclusion the necropsy was unsatisfactory in that it did not provide a cause of death of this animal.
PP160527	• Semi-obstructive nematode infection of the pulmonary vasculature multifocal marked chronic (Pseudalius inflexus) • Bilateral infestation of the ears with Stenurus minor • Lung: Pneumonia, granulomatous, multifocal, chronic, mild, associated with nematode infection. • Nematode infection of the right heart chamber mild chronic • Oesophageal erosion, focal, mild • Mesenteric lymph node: Lymphonodular granuloma, focal, chronic, marked. • Gastric erosion, focal, acute, mild.	• Cerebral meninges: Meningitis, lymphocytic, diffuse, mild. • Otitis media, necrotizing, suppurative, diffuse, chronic-active, marked, associated with Aspergillus sp.	• Pulmonary oedema (due to euthanasia) diffuse marked peracute	This juvenile male harbour porpoise in very poor nutritional condition was euthanized after four days in the rehabilitation centre. The most important finding was a unilateral middle ear infection which continued into the cerebral meninges and was associated with Aspergillus hyphae.

Table 2 Organs involved and etiological categories involved in significant diagnoses, per stranded harbour porpoise.




Organ involved in significant diagnoses
Other probable causes of stranding
Erasmus code number
Lung
Liver
Brain
Integument
Kidney
Muscle
Skeleton
Pancreas
Heart
Ear
Pharynx
Oesophagus
Stomach
Vasculature
Lymph nodes
Eyes
Sepsis
Orphaned neonate
Emaciated#
Unknown
PP061123.1
NON
NON
PP070221
PP070328
PP080403
NON
PP080701
PP081222
PP090626
PP110329
PP110711
PP120906.1
PP120906.2
PP120906.3
PP121031
PP121102
PP121130T
PP130829
PP140917

	Organ involved in significant diagnoses																Other probable causes of stranding			
Erasmus code number	Lung	Liver	Brain	Integument	Kidney	Muscle	Skeleton	Pancreas	Heart	Ear	Pharynx	Oesophagus	Stomach	Vasculature	Lymph nodes	Eyes	Sepsis	Orphaned neonate	Emaciated[#]	Unknown
PP151116																				
PP160527	WSD																			
PP030320	WSD																		EMA	
PP030919	WSD																			
PP031124.2	WSD																		EMA	
PP040627	WSD																		EMA	
PP060220	WSD					NON			NON											
PP060301	WSD							NON												
PP060327.1	WSD				NON		NON													
PP060327.4	WSD																			UNK
PP060524	WSD																			
PP070317	WSD																			
PP100105	WSD																		EMA	
PP100928.1	WSD																		EMA	
PP100928.2	WSD																	ORN		
PP110228	WSD																			
PP110928.1	WSD	NON				NON														

	Organ involved in significant diagnoses															Other probable causes of stranding			
Erasmus code number	Lung	Liver	Brain	Integument	Kidney	Muscle	Skeleton	Pancreas	Heart	Ear	Pharynx	Oesophagus	Stomach	Vasculature	Lymph nodes	Eyes	Sepsis	Orphaned neonate	Emaciated[#]	Unknown
PP110928.2	**WSD**			**NON**																
PP111219	**WSD**																		**EMA**	
PP120703	**WSD**																	**ORN**		
PP130204	**WSD**				**NON**	**NON**				**NON**										
PP130513	**WSD**																			**UNK**
PP140204	**WSD**																	**ORN**		
PP140627	**WSD**																		**EMA**	
PP150210	**WSD**																			**UNK**
PP160304	**WSD**				**NON**															
PP160331	**WSD**																			**UNK**

Legend for tables 1, 2 and 3

Yellow is Parasitic aetiology
Blue is Bacterial aetiology
Red is fungal aetiology
Black is viral aetiology
Grey is inflammation of unknown aetiology
ALP = Alveolar Proteinosis
NON = Non-inflammatory lesion
WSD= Lungs without significant diagnoses
L = 1-100 nematodes (both lungs
H = >100 nematodes (both lungs)
L/H= infection with unknown intensity
T = animal treated with anti parasitics
EMA= emaciated
ORN= Orphaned Neonate
UNK= No cause for stranding observed during necropsy
# emaciation was only severe lesion observed

Table 3: Nematode infections in juvenile harbour porpoises with and without severe pneumonia

			Nematode species			
				Halocercus		
Erasmus code number	Pneumonia caused by	Infectious organisms	*Stenurus minor* (<22 mm)	*invaginatus/ Torynurus convolutus* (30 - 70 mm)	*Pseudalius inflexus* (>100 mm)	age (days, est)
PP040324					**H**	296
PP050825.1				**H**	**H**	275
PP060501		Lungworm larvae				325
PP121130T				**L**	**H**	530
PP050825.2		Small lungworms (susp *Sten. minor*)	**L/H**			405
PP120906.3				**L**	**L**	345
PP041215		Bact: *Aeromonas sp.* 2 species		**H**	**H**	561
		Parasitic pneumonia: lungworm larvae				
PP060327.2		Bact: Coccoid bacteria		**H**	**L/H**	286
PP070221		Bact: *Actinobacillus delphinicola* and *Brucella sp.*		**H**	**H**	264
PP111219		Bact: *E. coli* and *S. maltophilia*		**H**		197
PP120906.2		Bact: *Streptococcus dysgalactiae*		**H**	**H**	372
PP040517		Fung: *Aspergillus fumigatus*	**L**	**L**	**L**	317
		Parasitic pneumonia: lungworm larvae.				
PP061122.1		Fung: *Aspergillus fumigatus*		**L**		353
		Bact: 4 species 3				
PP030405		specified: *Pseudomonas aeruginosa Streptococcus*				647

Legend for table 3 see underneath table 2

			Nematode species			
				Halocercus		
Erasmus code number	Pneumonia caused by	Infectious organisms	*Stenurus minor* (<22 mm)	*invaginatus/ Torynurus convolutus* (30 - 70 mm)	*Pseudalius inflexus* (>100 mm)	age (days, est)
		sp. Escherichia coli				
PP061122.2		Bact: *Escherichia coli*		**L**	**L**	490
PP070328		Bact: mixed coliforms		**H**		299
PP080701						294
PP140917		No growth				76
PP151116		No growth				137
PP120906.1		Fung: *Aspergillus fumigatus*			**L**	147
PP050610 T		Fung: *Aspergillus fumigatus*		**L (dead worms)**		325
PP080403		No growth		**H**		306
PP110711		Bact: *Brucella ceti* susp		**H**		1132
PP160527	**WSD**					331
pp030320	**WSD**			**H**		291
PP030919	**WSD**					104
PP031124.2	**WSD**					282
PP060301	**WSD**			**L**	**L**	266
PP060327.1	**WSD**					283
PP060327.4	**WSD**					253

Erasmus code number	Pneumonia caused by	Infectious organisms	Nematode species: *Stenurus minor* (<22 mm)	Nematode species: *Halocercus invaginatus/ Torynurus convolutus* (30 - 70 mm)	Nematode species: *Pseudalius inflexus* (>100 mm)	age (days, est)
PP060524	**WSD**					357
PP061123.1	**NON**					52
PP100105	**WSD**			**L/H**		215
PP100928.1	**WSD**					49
PP100928.2	**WSD**					37
PP110228	**WSD**				**L**	264
PP110928.1	**WSD**					17
PP110928.2	**WSD**			**L**		678
PP130204	**WSD**					216
PP140627	**WSD**					28
PP150210	**WSD**					240
PP160304	**WSD**			**L**		273
PP160331	**WSD**			**L**	**L**	272

Legend for table 3 see underneath table 2

Table 4 Nematode infections in adult harbour porpoises with and without severe pneumonia

			Nematode species		
Erasmus code number	Pneumonia caused by	Infectious organisms	*Stenurus minor* (<22 mm)	*Halocercus invaginatus/ Torynurus convolutus* (30 - 70 mm)	*Pseudalius inflexus* (>100 mm)
PP051106				**H**	**H**
PP090626				**H**	**L**
PP110329		Bact: *Actinobacillus delph*inicola		**H**	**H**
PP121031		Bact: *Salmonella sp.* (host adapted group B *Salmonella*)			**H**
PP081222		Bact: *Pseud. aeruginosa*		**L/H**	**L/H**
PP050208		Fung: *Aspergillus fumigatus*	**L**	**L**	
PP121102		Fung: *Aspergillus fumigatus*		**L**	**H**
PP130829		Fung: *Aspergillus fumigatus*		**L**	**L**
PP060227					**L**
PP060327.3					
PP050502 T	**AL P**				
PP060220	**WSD**			**H**	**L**
PP070317	**WSD**		**L/H**	**L/H**	**H**
PP130513T	**WSD**				

Legend for tables 4, see under table 2

Table 5 p-values according to Fisher's exact test (two-sided) comparing nematode infections in juvenile harbour porpoises with severe pneumonia to those in juveniles without severe pneumonia (n=20).

	Nematode species		
	Stenurus minor	**Mix *Torynurus convolutes* and *Halocercus sp.***	***Pseudalius inflexus* (bronchi, lung)**
Juveniles with severe pneumonia (n=21/22)*	$p \geq 0.47$	$p \leq 0.008$	$p \leq 0.002$
Juveniles with a severe parasitical pneumonia (incl. combined parasitic-fungal-bacterial infections) (n=13)	$p \geq 0.36$	$p \leq 0.003$	$p \leq 0.00006$
Juveniles with a severe parasitic pneumonia (n=5/6)*	$p = 0.2$	$p \leq 0.17$	$p = 0.005$
Juveniles with a severe bacterial pneumonia (incl. combined bacterial and parasitic infections) (n=12)	$p \geq 0.27$	$P = 0.008$	$p \leq 0.006$
Juveniles with only a severe bacterial pneumonia (n=5)	$p = 1.0$	$p > 0.54$	$p = 0.25$
Juveniles with a severe fungal pneumonia (incl. mixed fungal and parasitic infections) (n=3/4)*	$p = 0.45$	$p = 0.007$	$p = 0.00009$

Values indicate the outcome of the test comparing the sample mentioned in the left-hand row to the sample of animals without pneumonia. The top left value, for example, tells us that the statistical chance that juveniles with a pneumonia and without a pneumonia came from a population with the same prevalence and intensity of *Stenurus minor* infection is 0.47.

Larger or smaller than p values indicate animals with unknown number of parasites were in the test. For these animals' calculations were done with all possible combinations of light or heavy infections. (in red: $p < 0.05$ = significant difference in lungworm infection compared to animals without pneumonia).

Animals which have received anti-parasitic treatment were excluded. * one anti-parasiticum treated juvenile animal with a light infection of dead worms (mix *Torynurus convolutes/ Halocercus sp.*) This single result was taken into consideration for the statistical analysis and tested as an infection of unknown intensity.

All untreated adult animals with parasitical (n=2), bacterial (n=3), fungal (n=3) or without pneumonia (n=2) had light or heavy infections with both *Torynurus convolutus/Halocercus sp.* and *Pseudalius inflexus*. Apart from one animal with a bacterial infection where no *Torynurus convolutus* or *Halocercus sp.* was observed. Statistical analysis comparing animals with and without pneumonia in relation to parasite load, was therefore not informative. For *Stenurus minor* infections infestation in animals without pneumonia was higher compared to animals with bacterial or fungal pneumonia.

Table 6 Comparison of prevalence and abundance of ***Pseudalius inflexus*** infections in the pulmonary vasculature of juveniles and adult harbour porpoises.

		No. of animals (percentage of animals) with estimated parasite abundance of:			
Age category	**No. of animals infected (prevalence of infection)**	**1 – 10**	**11 – 100**	**101 – 1000**	**Unknown**
Juveniles (n = 41)	19 (46 %)	2 (10 %)	10 (50 %)	2 (10 %)	5 (25 %)
Adults (n = 14)	10 (71 %)	4 (40 %)	1 (10 %)	2 (20 %)	3 (30 %)

Table 7 Comparison of prevalence of gastrointestinal parasites in juvenile and adult harbour porpoises.

	Parasite species and location of infection				
Age category	***C. oblongata Liver***	***C. oblongata Pancreas***	***A.Simplex Fore stomach***	***P. Gastrophilus Fundic stomach***	***D. stemmacephalum Intestines***
Juveniles (n=41)	5	0	2	4	1
Adults (n=13)	6	1	4	0	0
P values	**0.02**	0.24	**0.02**	0.56	1

Numbers are numbers of animals

P values according to fisher test. P< 0.05 indicates significant difference in prevalence

Table 8 Comparison of prevalence and abundance of different parasite species in the digestive tracts of juvenile and adult harbour porpoises

Age category	No. of animals (percentage of animals) infected, per organ and corresponding parasite species				
	Pancreas *(Campula oblonga)*	**Liver *(Campula oblonga)***	**Forestomach *(Anisakis simplex)***	**Pyloric stomach *(Pholeter gastrophilus)***	**Intestine (cestode, likely *Diphyllobothrium stemmacephalum)***
Juveniles (n = 41)	0 (0 %)	5 (12 %)[u]	2 (5 %)[l]	4 (10 %)[u]	1 (2 %)[l]
Adults (n = 13)	1 (8 %)[u]	6 (46 %)[u]	4 (31 %)[h]	0 (0 %)	0 (0 %)

u, parasite abundance unknown
l, parasite abundance light
h, parasite abundance high

Table 9 Overview of lesions, diagnosis and most prominent clinical signs.

Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP030320	Emaciation		Tachypnoea (16/min), hypothermic, vomiting	
PP030405	Lungs	Pyogranulomatous pneumonia multifocal chronic severe, eyes (corneal perforation)	Nervous: bumped into wall	
PP030919	Integument	dermatitis, fibrinosuppurative, superficial, diffuse, acute, marked, with bulla formation	increase in temperature, intermittent vomiting and increase in breathing frequency	Animal developed acute bullae on dorsal side of body
PP031124.2	Emaciation		Vomiting, passive unable to keep afloat	
PP040324	brain, lungs	Bronchopneumonia, histiocytic, multifocal, chronic-active, marked, suppurative, associated with lungworm infection 1. meningitis, non-suppurative, segmental, chronic, moderate (cerebrum) 2. neuronal necrosis, segmental, moderate (cerebrum)	increasing tachypnoea	
PP040517	lungs, brain metastases suspected based on symptoms	1. Bronchopneumonia, multifocal, chronic-active, moderate, suppurative, associated with lungworm larvae 2. pneumonia, necrotizing, focal, acute, mild,	Nervous: epileptiform attacks	
PP040526	lungs	Interstitial pneumonia, suppurative, multifocal, acute, moderate	respiratory; bradypnea, respiratory arrest	
PP040627	susp reaction to fish porridge no sign path observed		Abdominal: severe continuous cramping with a sharp rise in BF	
PP050208	lungs	Interstitial pneumonia, suppurative, multifocal, acute, moderate	Unable to dive	

Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP050502 T	lungs, kidneys, integument	1. Bronchitis, lympho-plasmacytic, diffuse, subacute, moderate, suppurative, 2. Pneumonia, histiocytic, multifocal, chronic, moderate to severe, with abundant foamy material 3. nephritis, suppurative, focal, acute, mild containing small round pink bodies about 0.5 µm diameter (alveolitis) 4. Dermatitis, multifocal, suppurative, superficial, acute, moderate, associated with bacterial infection	Kidney failure as was evident by marked increase of urea, creatinine and Sodium, brown urine, **loss of body weight and anorexia**	
PP050610	lungs, muscles, heart, skeleton	1. Lung: bronchopneumonia, necrotizing, haemorrhagic, granulomatous, multifocal or diffuse, chronic, marked 2. Pleura: pleuritis, granulomatous, multifocal or diffuse, moderate or marked. 3. Right and left heart ventricle: epicarditis and myocarditis, granulomatous, necrotizing, multifocal, chronic, moderate. 4. Vertebra (tail stock): osteomyelitis, pyogranulomatous, focal, chronic, marked, with exophytic bone formation.	High BF, high PR, clear infect resp tract	
PP050825.2	lungs, brain	1. Bronchopneumonia, pyogranulomatous, multifocal, chronic, associated with nematode infection (probably Stenurus minor). 2. Encephalitis, lymphocytic, locally extensive, subacute, with neuronal necrosis and intranuclear inclusion bodies (herpesvirus).		She lifts her head out of the water and the respiratory muscles make a large effort to exhale. Gastric stasis occurs and at day 7 she starts to vomit
PP060220	muscles, heart	1. Right ventricular wall thinning. 2. skeletal muscle degeneration, diffuse, subacute, marked.	heavy cramps and forced breathing in final stage	spasms as from receiving an electric shock, disoriented swimming (bumping against the wall) and sporadic vomiting

Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP060227	brain, lungs	1. Bronchopneumonia, suppurative, locally extensive, acute, moderate. 1. Cerebrum: encephalitis, lymphocytic, diffuse, subacute, moderate, with multifocal gliosis, perivascular cuffing, and oedema. 2. Cerebellum: meningo-encephalitis, lymphocytic, subacute, moderate, with multifocal gliosis and loss of Purkinje cells. 3. Cervical spinal cord: myelitis, lymphocytic, diffuse, subacute, moderate.	lethargic curved position, final morning sharp increase BF, agitated.	
PP060301	pancreas, emaciation	pancreatic duct hyperplasia/occlusion, emaciation	Initially none, in final stage vomiting and increased BF	
PP060327.1	musculoskeletal trauma	musculoskeletal trauma		
PP060327.2	lungs	Pneumonia, multifocal, chronic, moderate, pulmonary abscesses associated with large and small nematodes		
PP060327.3	lungs, liver, cardiac	1. Interstitial pneumonia, histiocytic, locally extensive, chronic, moderate, associated with unknown organisms. 2. Tracheo-bronchitis, lymphoplasmacytic, diffuse, subacute, marked, suppurative, superficial. 3. Hepatitis, necrotizing, multifocal, acute, marked. 4. Right ventricular wall thinning.	sporadic vomiting, arching, less active	Ronchi in left bronchi
PP060327.4	Brain suspected		• Increased breathing frequency • Vomiting • Stasis of gastric content • Exudate occasionally with blood expired from the blowhole • Body tremor • Cramps • Forceful difficult exhalation (appears to choke)	

Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP060524	brain, ear	1. Dura mater: pachymeningitis, necrotizing, suppurative, locally extensive, acute, marked, associated with fungal hyphae (Aspergillus sp.). 2. Cerebellum: panencephalitis, pyogranulomatous, haemorrhagic, necrotizing, locally extensive, associated with fungal hyphae (Aspergillus sp.) and mixed bacterial infection. 3. Otitis media purulent subacute to chronic diffuse severe.	At first the animal appeared to swim uncoordinated with the tail making a cork screw type of motion. The pupil reflex of the right eye was slow and a slight vertical nystagmus of the right eye was visible.	
PP061122.1	lungs	1. Bronchopneumonia, multifocal, chronic, mild to moderate, pyogranulomatous, 2. Bronchopneumonia, multifocal, chronic, moderate, pyogranulomatous, necrotizing	Increased BF, pulse, Vomiting. Blood loss from blowhole (once) and melaena, emaciated and very anaemic. Occasional exaggerated breathing movements	
PP061122.2	lungs, sepsis	1. Bronchopneumonia, multifocal, subacute to chronic, severe, suppurative. 2. Myositis, suppurative, multifocal, acute, moderate (consistent with Escherichia coli infection). 3. Fasciitis, suppurative, focal, acute, moderate (consistent with Escherichia coli infection).	initially active and with good appetite afterwards listing, varying appetite, lethargy	slightly elevated BF (22 - 28/5min)
PP070221	lungs	1. Bronchopneumonia, multifocal, lymphocytic and eosinophilic, chronic, moderate, associated with nematode infection. 2. Bronchopneumonia, multifocal, suppurative, acute, moderate, associated with bacterial infection.	slight tachypnoea 9/min to 6/min a few hours after arrival	slight tachypnoea 9/min to 6/min a few hours after arrival
PP070317	liver	1. Hepatic necrosis, periacinar, extensive, acute, marked associated with a moderate icterus 2. Hepatic lipidosis, diffuse, acute, moderate	At first inactive and floating, then swimming but not diving on final day diving. Low body temperature. Final day vomiting.	severe leukopenia final two days
PP070328	lungs	Bronchopneumonia, diffuse, acute, moderate, necrotizing, suppurative, associated with bacterial infection (mixed coliforms).	Increased BF (died during transport)	

Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP080403	lungs, integument	1. Bronchopneumonia, locally extensive, acute, marked, suppurative (too acute to explain emaciation). 2. Severe trauma to blowhole and eyelids (necessitating euthanasia)	none noted apart from the observation that blowhole could not be closed	
PP080701	lungs, sepsis, liver, pancreas	1. Interstitial pneumonia, diffuse, acute, marked, haemorrhagic, (post rehab) 2. Pleuritis bilateral focally extensive fibrinopurulent acute moderate (post rehab) 3. Pneumonia, lymphohistiocytic, locally extensive, chronic, marked. 1. Liver: bile duct hyperplasia, diffuse, marked. 2.Pancreatic angitis, suppurative, necrotizing, chronic-active, multifocal, marked 3. Pancreatic duct hyperplasia, focal, moderate.	No clinical signs apart from increasing liver enzymes during rehab. Final day anorexia, vomiting, coughing (blood), fever, increased BF	
PP100105	lungs (in reh acquired), emaciation, muscles	Bronchopneumonia, diffuse, chronic-acute, marked, suppurative, granulomatous, associated with mixed bacterial and yeast-like (?) infection and aspiration of food remains marked. 2. Muscle degeneration, multifocal, acute, moderate.	tachypnoea, squeaks on auscultation, dark urine high bun while normal creatinine	
PP100928.1	emaciation			
PP100928.2	emaciation		drop in albumin and tp despite hand rearing formula, increasing weakness and lethargy	
PP110228	lungs (acquired in rehab), oesophagus	1. Oesophagitis ulcerative multifocal to confluescent acute marked 2. Pneumonia, diffuse, (per) acute, mild, suppurative, associated with microthrombi.	Passive, blood loss upon expiration with vomitus and with defaecation	
PP110711	lungs, liver, emaciation	1. Pneumonia, pyogranulomatous, locally extensive, chronic marked. 2. Hepatocytic degeneration, random, multifocal, acute, moderate, with neutrophil infiltration and hepatocytic necrosis.	tachypnoea (7 -8,5/ min average at times extr high) Signs of resp distress (head out of water tail bending) forced audible gurgling breathing. Elevated temp (38 to 38,3) lack of appetite, moderate leukopenia	

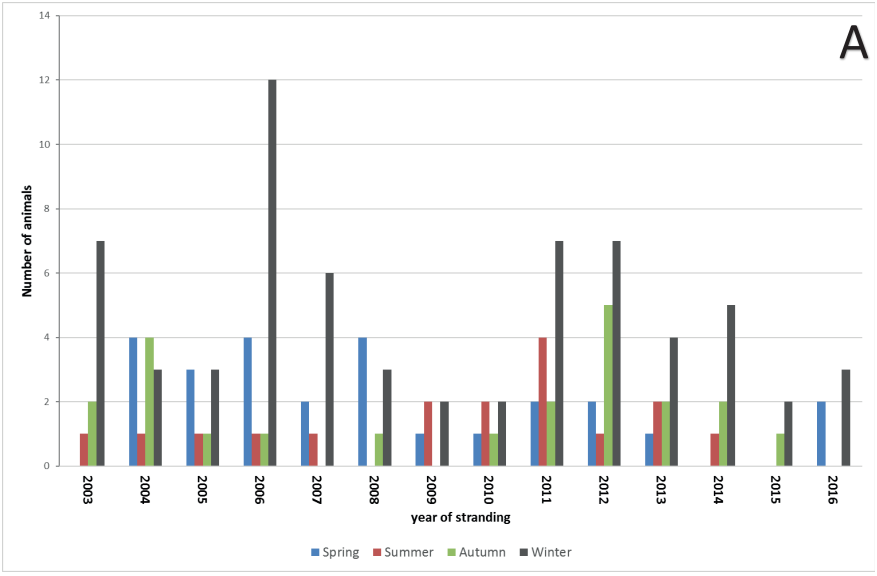
Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP110928.1	liver, kidney	1. Hepatocytic degeneration, random, multifocal, acute, moderate, with neutrophil infiltration and hepatocytic necrosis. 2. Renal tubular epithelial cell vacuolation, diffuse, marked.	defence musculaire, vomiting when given food,	bloodwork indicates kidney failure
PP111219	lungs (aq in rehab), lungs, emaciation	1. Bronchopneumonia, haemorrhagic, suppurative, diffuse, acute, marked, associated with bacterial infection, suspected aspiration pneumonia 2. Lung: alveolitis, pyogranulomatous or granulomatous, multifocal, chronic, mild or marked, associated with nematode infection	final day diff moist breathing, final hours sharp increase in breathing rate 18/min	
PP120906.1	lungs, brain	1. Pneumonia, multifocal to coalescing, chronic, marked, pyogranulomatous. 2. Encephalitis, pyogranulomatous, multifocal, chronic, moderate.	dyspnoea with forced laboured breathing with gurgling sounds and exudate and accompanied by body turns (curved cramps) to the right side. Breathing frequency was increased	sporadic vomiting and white and red bits of exudate sporadically upon expiration
PP120906.3	lungs, liver, brain	1. Interstitial pneumonia, suppurative, histiocytic, locally extensive, chronic, moderate, suppurative 2. bronchopneumonia, granulomatous, multifocal, chronic, moderate. 3. Liver: hepatitis, necrotizing, multifocal, acute, marked. 4. Cerebrum: polioencephalitis, multifocal, mild.	The last day the animal showed less to absent appetite, had severe hypothermia (33 degrees Celsius) swam disorientated against the wall and had laboured difficult breathing with vertical body rises from the water for inspiration.	Blood values on the last day (only) showed increase in WBC, drop in Ht and Hgb and a slight hypernatremia.
PP121102	lungs	Pneumonia, multifocal, chronic, marked, granulomatous	Passive low BF, very frequent coughing, passive only mild inflammatory markers rise in blood work, difficult breathing	Ronchi in bronchi (left side mainly)
PP121130T	lungs, vasculature, skin	1. Bronchopneumonia, locally extensive, chronic, moderate, pyogranulomatous. 2. Forestomach, jejunum, colon: vasculitis, leukoclastic, haemorrhagic, multifocal, chronic-active, marked. 3. Dermatitis, pyogranulomatous, multifocal, chronic, marked, with epidermal hyperplasia, keratin pearls, and bacterial infection.	Last 48 hours passive and stiff, gained one kilo (ascites possibly) lowered breathing rate.	High to very high WBC not reacting to antibiotic therapy

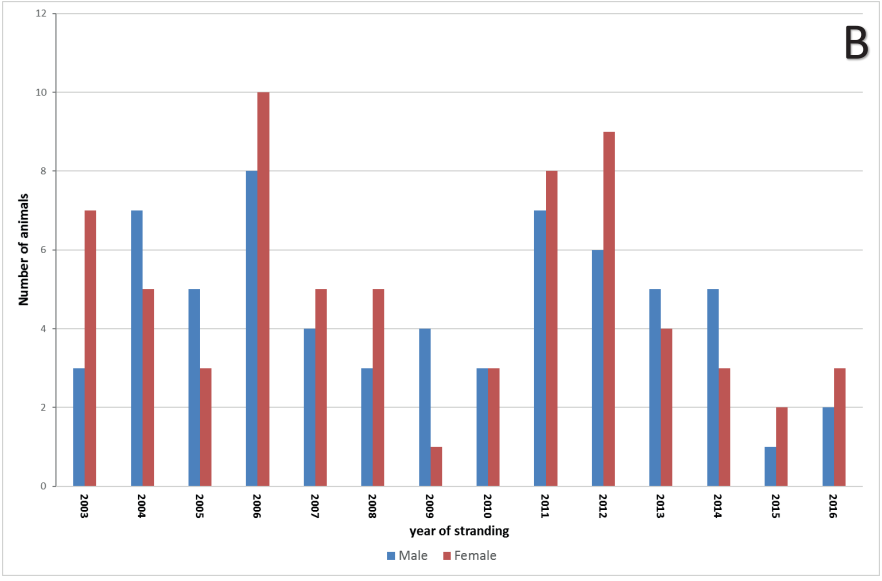
Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP130204	kidney, bladder, muscles, heart	1. Kidney: urolithiasis, mild, 2. subserosal haemorrhages on urinary bladder. 3. muscle degeneration, locally extensive, acute to subacute, marked. 4. Right heart: myocardial oedema, multifocal, acute, mild.	passive, strong defence musculaire, regular vomiting	
PP130513	unknown	unknown	Unable to swim or dive throughout its six months stay in the rehab centre. Necropsy did not provide an explanation for this sign.	
PP130829	lungs, pharynx	1. Lung: pulmonary abscesses, multifocal, chronic, marked, associated with Aspergillus fumigatus infection. 2. Lung: bronchopneumonia, pyogranulomatous, coalescing, chronic, marked, probably associated with Aspergillus fumigatus infection. 3. palatal abscess, focal, chronic, marked.	Mucous exudates, and gurgling moist sounds with respiration, multiple abdominal cramps	
PP140204	emaciation			
PP140627	emaciation			
PP140917	lungs, skin	1. Bronchopneumonia, suppurative, multifocal, acute to subacute, moderate to marked. 2. Skin of snout: Dermatitis, suppurative, necrotizing, locally extensive, superficial, acute, marked.		
PP150210	heart (but not reason for stranding), unknown	Ventricular septal defect, congenital	vomiting, constipation, coughing, leucocytosis	
PP151116	lungs, skin, bones, emaciation	1. Bronchopneumonia, fibrinopurulent, unilateral, locally extensive, acute, moderate. 2. Puncture wounds with suppurative dermatitis, multifocal, acute to subacute, superficial, mild. 3. Tail vertebra: Puncture wound with suppurative osteitis, focal, acute, superficial, mild.		

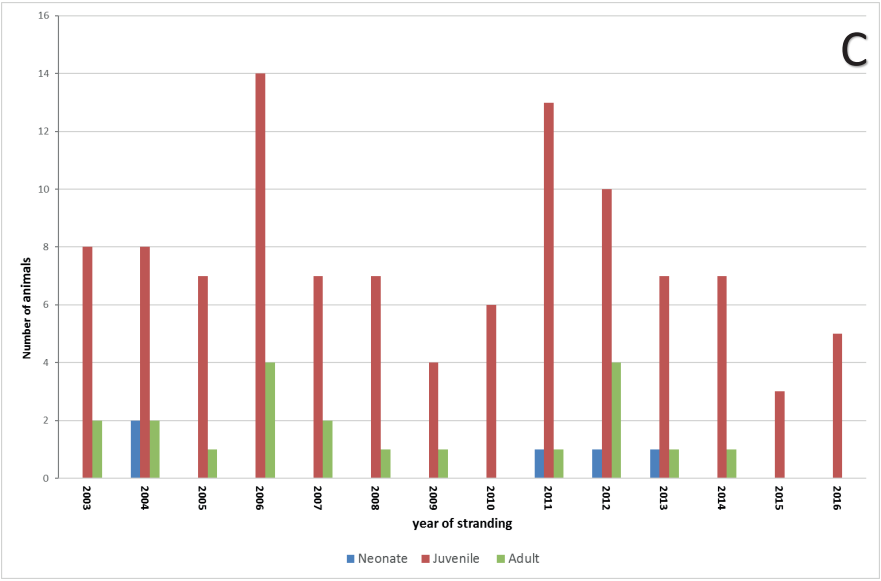
Erasmus code number	Organs affected by lesions which contributed to stranding, emaciation, suspected aetiology	diagnosis	Most prominent clinical signs	Other and or remarkable signs
PP160304	kidney (protein loss), emaciation	Approximately half of the glomeruli have a distended capsule of Bowman, which is filled with protein. Many cortical tubuli also are filled with granular eosinophilic material.	shaking when out of water, vomiting and continuous weight loss	
PP160331	none	none	Increased cardiac frequency (160/min no resp arrythmia), drop in albumin and TP despite good food intake, with a rise in Na and Cl indicating kidney failure and or vomiting (although not observed). Final day swimming into wall and unable to maintain upright position.	
PP160527	meninges, ear, emaciation	1. Otitis media, necrotizing, suppurative, diffuse, chronic-active, marked. 2. Cerebral meninges: Meningitis, lymphocytic, diffuse, mild.	Increased cardiac frequency (160/min no resp arrythmia), drop in albumin and TP despite good food intake, with a rise in Na and Cl indicating kidney failure and or vomiting (although not observed). Final day swimming into wall and unable to maintain upright position.	

ADDITIONAL FILE 2

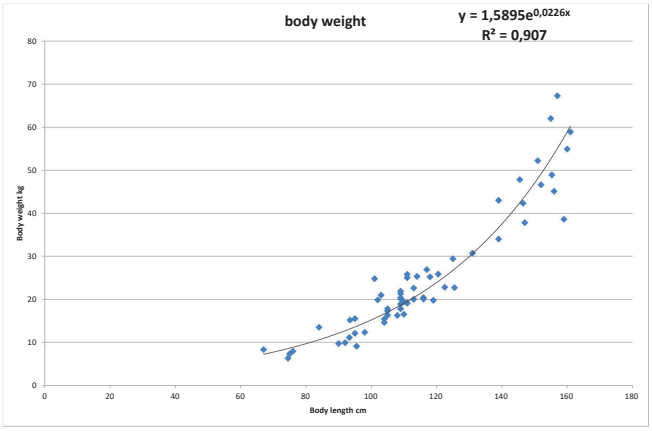
Number of admissions per year variation according to season (A), gender (B) and age class (C)

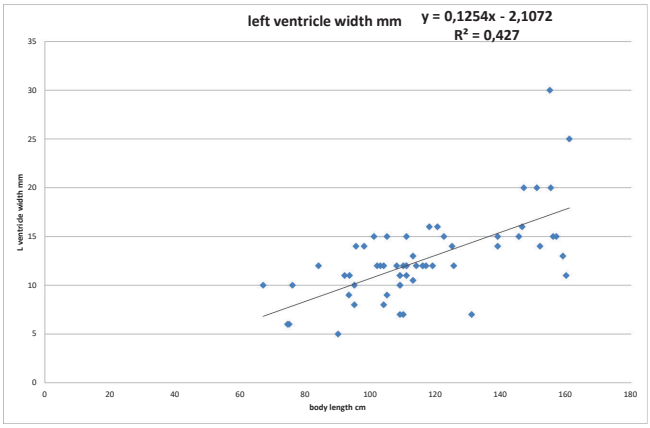


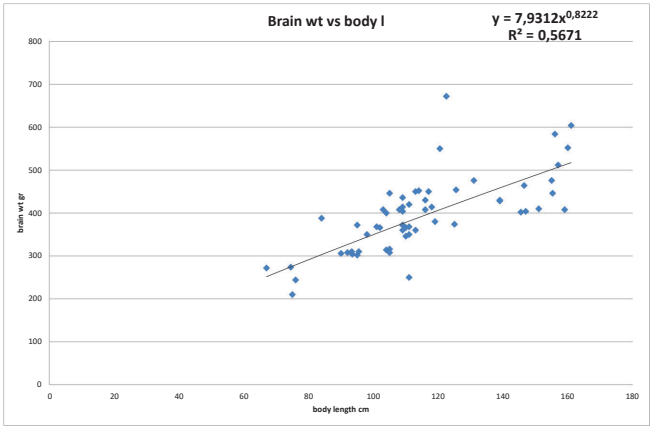


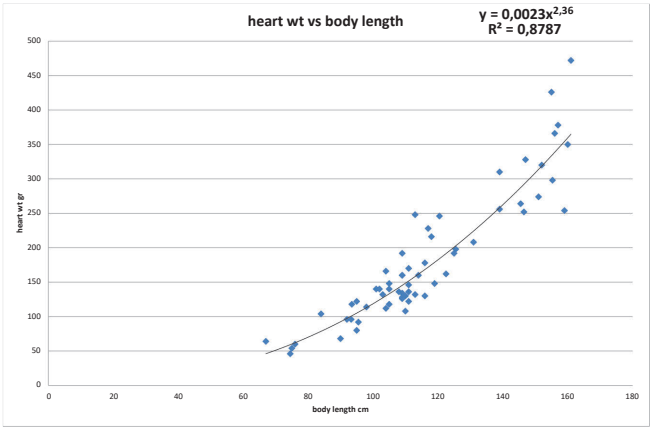

C
Number of animals
16
14
12
10
8
6
4
2
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
year of stranding
Neonate
Juvenile
Adult

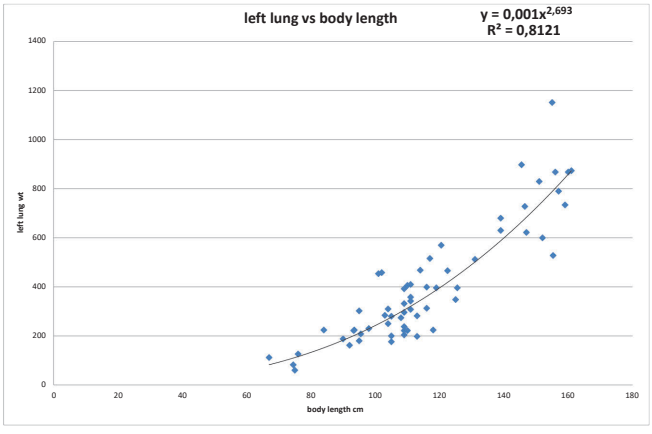
ADDITIONAL FILE 3

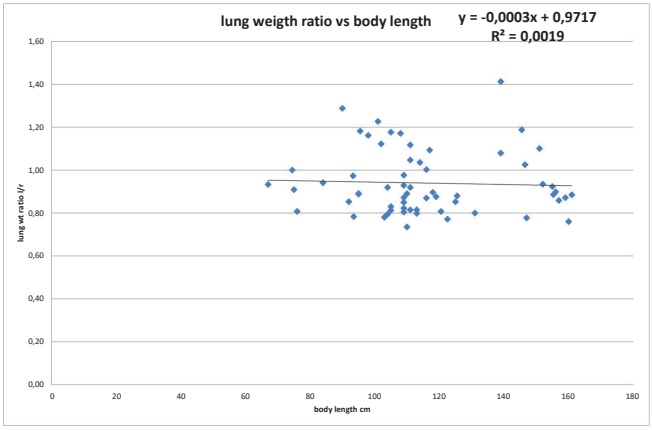


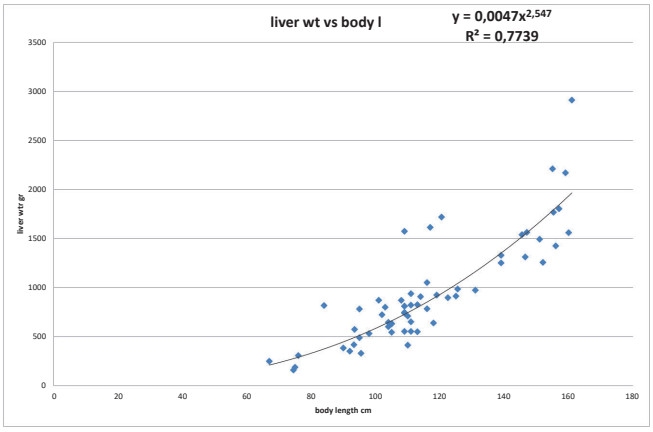


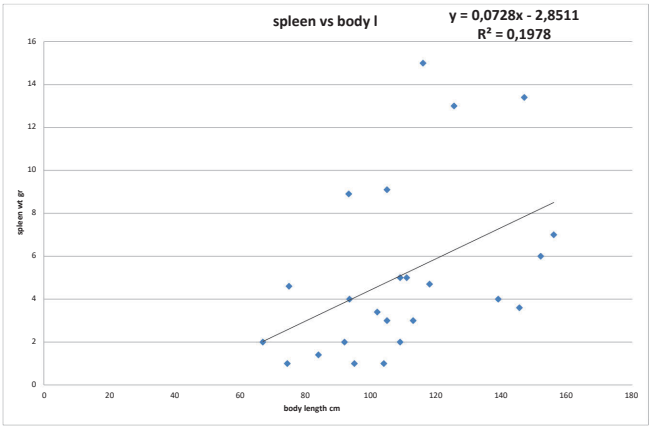

Brain wt vs body l
y = 7,9312x^0,8222
R² = 0,5671
brain wt gr
body length cm
0
100
200
300
400
500
600
700
800
0
20
40
60
80
100
120
140
160
180

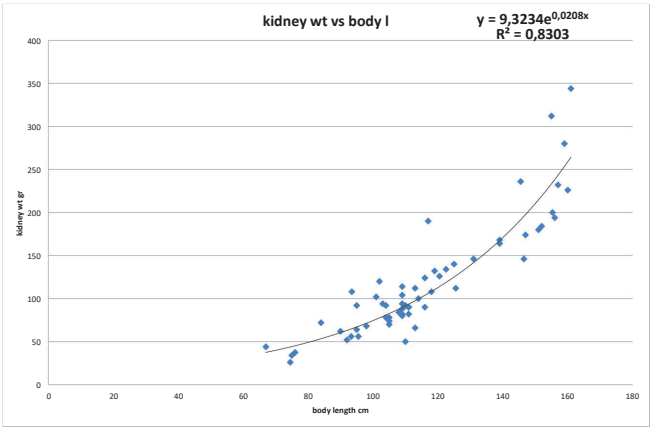

heart wt vs body length
y = 0,0023x^2,36
R² = 0,8787
heart wt gr
body length cm
0
50
100
150
200
250
300
350
400
450
500
0
20
40
60
80
100
120
140
160
180

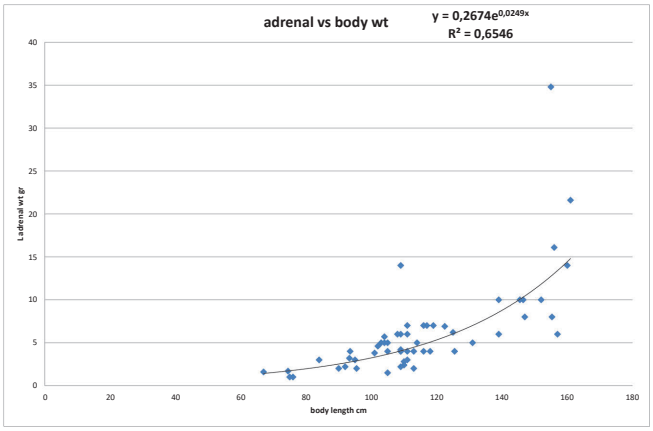

left lung vs body length
y = 0,001x^2,693
R² = 0,8121
left lung wt
body length cm
0
200
400
600
800
1000
1200
1400
0
20
40
60
80
100
120
140
160
180

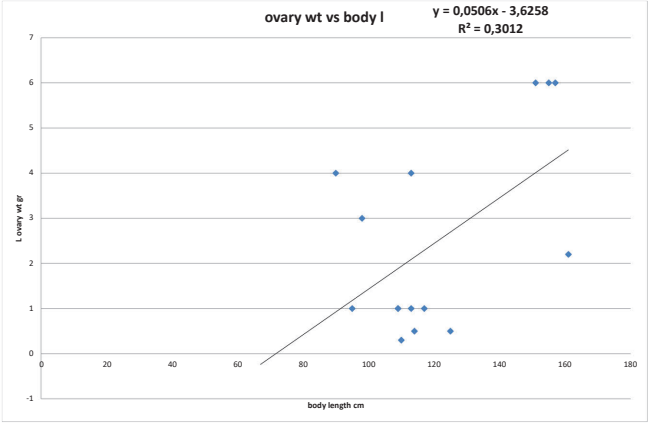

lung weigth ratio vs body length
y = -0,0003x + 0,9717
R² = 0,0019
lung wt ratio l/r
body length cm

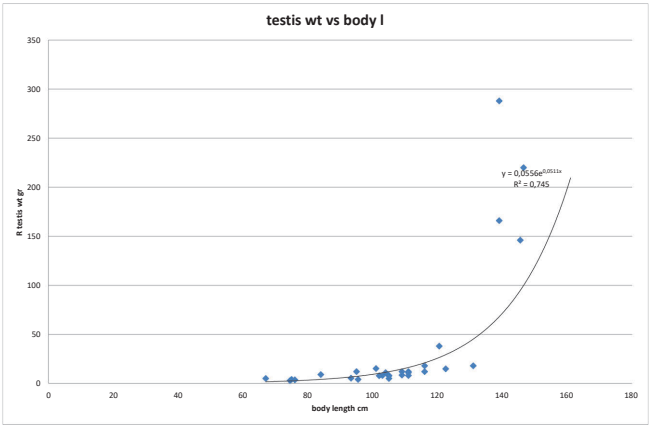

liver wt vs body l
y = 0,0047x^2,547
R² = 0,7739
liver wtr gr
body length cm

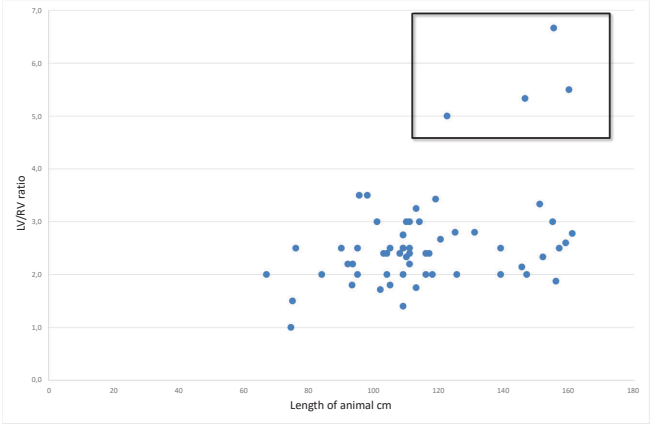

spleen vs body l
y = 0,0728x - 2,8511
R² = 0,1978
spleen wt gr
body length cm


kidney wt vs body l
y = 9,3234e^0,0208x
R² = 0,8303
kidney wt gr
body length cm
0
50
100
150
200
250
300
350
400
0
20
40
60
80
100
120
140
160
180


adrenal vs body wt
y = 0,2674e^0,0249x
R² = 0,6546
L adrenal wt gr
body length cm
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120
140
160
180


ovary wt vs body l
y = 0,0506x - 3,6258
R² = 0,3012
L ovary wt gr
body length cm
-1
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
160
180


testis wt vs body l
R testis wt gr
body length cm
y = 0,0556e^0,0511x
R² = 0,745


LV/RV ratio
Length of animal cm

harbour porpoise, Danish coatal wateres
Jakob Højer Kirstensen

3

AN EVOLUTIONARY DIVERGENT PESTIVIRUS LACKING THE NPRO GENE SYSTEMICALLY INFECTS A WHALE SPECIES

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ABSTRACT

Pestiviruses typically infect members of the order Artiodactyla, including ruminants and pigs, although putative rat and bat pestiviruses have also been described. In the present study, we identified and characterized an evolutionary divergent pestivirus in the toothed whale species, harbor porpoise (*Phocoena phocoena*). We tentatively named the virus Phocoena pestivirus (PhoPeV). PhoPeV displays a typical pestivirus genome organization except for the unique absence of N^{pro}, an N-terminal autoprotease that targets the innate host immune response. Evolutionary evidence indicates that PhoPeV emerged following an interspecies transmission event from an ancestral pestivirus that expressed N^{pro}. We show that 9 % (n = 10) of stranded porpoises from the Dutch North Sea coast (n = 112) were positive for PhoPeV and they displayed a systemic infection reminiscent of non-cytopathogenic persistent pestivirus infection. The identification of PhoPeV extends the host range of pestiviruses to cetaceans (dolphins, whales, porpoises), which are considered to have evolved from artiodactyls (even-toed ungulates). Elucidation of the pathophysiology of PhoPeV infection and N^{pro} unique absence will add to our understanding of molecular mechanisms governing pestivirus pathogenesis.

INTRODUCTION

Pestiviruses are enveloped viruses that belong to the genus Pestivirus within the family Flaviviridae. They have a wide host range and are responsible for significant levels of morbidity and mortality worldwide in mammals of the order Artiodactyla, which include ruminants and pigs. The typical pestivirus species are classical swine fever virus (CSFV) in pigs, bovine viral diarrhoea virus 1 and 2 (BVDV-1 and BVDV-2) in cattle, and border disease virus (BDV) in sheep [178]. Atypical pestiviruses have also been found in wild ruminants, such as antelopes and giraffes [179, 180]. In the past two decades, improved technology and better surveillance have facilitated the detection of new members of the genus Pestivirus, in some cases leading to expansion of known host species [181]. In pigs, three novel pestiviruses evolutionarily distant to CSFV have been identified: Bungowannah pestivirus, atypical porcine pestivirus (APPV), and most recently the lateral shaking inducing neurodegenerative agent (LINDA) virus. Recent metagenomics studies have indicated that pestiviruses are not restricted to Artiodactyla species, as putative bat pestiviruses (BatPeV) and rat pestiviruses have been discovered [51, 52], along with a pestivirus-like virus in a soybean cyst nematode [182].

Pestiviruses have two biotypes, cytopathic (cp) and non-cytopathic (ncp) viruses. The latter generate persistent infections without overt damage and are the most common forms found in the field [49]. Infected animals display mostly inapparent or mild symptoms. However, animals with acute infection can develop severe disease, characterized by

hemorrhages, respiratory failure, gastrointestinal problems, and central nervous system disorders [181]. Recombination events upstream viral protein NS3 appear to trigger the cleavage of NS2-NS3 by autoprotease activity exerted by NS2, after which the virus does become cytopathic [183]. The genome of pestiviruses consists of a single molecule of positive-sense single-stranded RNA of about 12.3 kb that encodes one long open reading frame of about 3900 amino acids (aa) [184]. Pestiviruses are distinguished from other flavivirus genera by the unique presence of Npro and Erns, which are both essential in antagonizing mediators of the host innate immune response [49]. The autocatalytic N-terminal protease Npro is present at the beginning of the polyprotein and targets the host interferon regulatory factors 3 and 7 [185, 186]. The glycoprotein Erns possesses RNase activity influencing the interferon response.

Investigation into strandings of harbor porpoises (Phocoena phocoena) along the Dutch North Sea coast over a 15 year period was undertaken to identify morbidity and mortality factors, including possible underlying viral aetiologies. Previous studies have reported a limited number of novel viruses in these marine mammals that are heterologous to viruses of terrestrial mammals, including porpoise morbillivirus [187], norovirus [43], herpesviruses [155] and adenovirus [188]. We report the identification and characterization of a novel divergent pestivirus and demonstrate that the presence of Npro is not a prerequisite for a pestivirus to infect its host.

MATERIALS AND METHODS

Animals and post-mortem examination

The samples used in this study were obtained from wild harbor porpoises that had stranded dead or alive along the Dutch North Sea coast. The live ones had been taken to the Dutch rehabilitation centre SOS Dolfijn (application number FF/75/2012/036) where they survived for variable periods of time. Animals that died or had to be euthanized were autopsied at Erasmus Medical Center. Tissue samples were fixed in 10 % neutral-buffered formalin, embedded in paraffin and used for diagnostic purposes to determine the cause of stranding. Additional samples from the same organs were also frozen at –80°C for virological investigations.

Next generation sequencing

Lung and brain tissue samples of three harbor porpoises (NS170385-87), suspected with encephalitis upon clinical observation by a veterinarian and diagnosed postmortem by histological examination, were processed for Next generation sequencing (NGS). Briefly, 25-105 mg of tissue were lysed in 500 µL of PBS using ceramic beads in a FastPrep-24 5G homogenizer (MP Biomedical), followed by 3× freeze/ thaw cycles. Homogenates were

centrifuged and passed through a 0.45 µm filter. RNA was isolated using TRI- zol (Thermo Fischer Scientifics, Waltham, MA, USA) and transcribed to cDNA using a mix of random and non-ribosomal hexamers [189] by Superscript IV (Thermo Fischer Scientifics). Second strand cDNA was generated by Klenow fragment (New England Bio- lab [NEB], Ipswich, MA, USA). Random amplification of samples was performed following a sequence-independent, single-primer amplification protocol [190]. PCR products were purified and the DNA library was prepared according to NEBNext® Ultra™ II DNA Library Prep Kit protocol (NEB) and subsequently sequenced on an Illumina MiSeq system with the MiSeq Reagent Kit v3 (2 × 300 bp paired-end; Illumina).

Generation of full-length genome sequence

NGS raw data were analysed using a previously developed metagenomics pipeline as described [191]. Quality-trimmed reads were mapped to DNA and peptide viral sequences database retrieved from GenBank using Bowtie v2.2.9 [192] and Pauda v1.0.1 [193]. Quality trimming of raw data and de novo assembly of contigs larger than 500 bp was followed using the software CLC Genomics Workbench v11 (CLC Bio, Aarhus, Denmark). Contigs were also mapped against non- redundant protein sequences database (blastx) using the same software. All contigs with similarity hits to pestiviruses were retrieved and an assembly was performed using SeqMan Pro (LaserGene software package, DNAStar, Madison, WI). To confirm genome sequence generated by NGS data, primers were designed to amplify the complete genome of NS170385-Lung (Table S1, Supplementary Information). A rapid amplification of cDNA ends (RACE) protocol to determine 5′ genome end was used. Briefly, RNA was polyadenylated with a poly(A) polymerase (NEB) and transcribed to cDNA using a poly(T) adaptor flanking the 5′ end. A PCR was then performed with primers designed to target the newly inserted poly(T) tail as well as the 5′ region of the novel pestivirus genome generated by NGS data (Table S1, Supplementary information).

Phylogenetic analyses

Complete genome sequences of 53 pestiviruses representative of all identified pestivirus species found to date (A-K), were retrieved from GenBank database (Table S2, Supplementary Information). Alignment of nucleotide and protein sequences was conducted by MAFFTv7 [194]. Phylogenetic trees of the complete polyprotein and partial PhoPeV nucleotide genomes (5′UTR, C, Erns, and E2) were calculated using the maximum likelihood method in MEGA7.0 [195] with 1000 bootstraps. According to the Bayesian information criterion, LG + G + F was selected as best-fit model for the complete polyprotein, whereas TN93 + G was selected as best-fit model for the partial nucleotide genomes.

Histopathological analysis and in situ hybridization

After fixation in 10 % neutral-buffered formalin and embedding in paraffin, tissue sections from animals NS170385 and NS170386 were stained with haematoxylin and eosin for histopathological evaluation or with in situ hybridization (ISH) as described previously [155]. A probe targeting specific PhoPeV NS2-NS3 region was designed by Advanced Cell Diagnostics (Hayward, California, USA). ISH was performed using RNAscope 2.0/2.5 assay kit (Advanced Cell Diag- nostics, Inc.) following manufacturer instructions for FFPE samples. In brief, 5-µm-thick tissue sections were deparaffinised in xylene and dehydrated in 100 % ethanol. Slides were next pretreated to allow access to target RNA. The probe was subsequently added to slides and hybridized for 2 h at 40°C with six subsequent amplification steps. Signal was visualized with Fast Red. The section was counterstained with haematoxylin and mounted with Ecomount.

Screening of PhoPeV in harbor porpoises

A PhoPeV-specific real-time reverse transcription PCR (qRT-PCR) was developed to screen for the novel pestivirus in stranded harbor porpoises from the North Sea. The primers and probe were designed to target the NS3 region of PhoPeV, with 5′-aaccatctgagtgtgaccttgagtc-3′ as forward primer, 5′-tcaatcaaccttcttggtagctcagtg-3′ as reverse primer, and 5′-tttaaacaagtgaccctggccaccgg-3′ as probe labelled with FAM-BHQ-1. Samples were homogenized, centrifuged and supernatants taken for RNA extraction. Automated sample processing was performed with a QIAcube instrument using the QIAmp Viral RNA Mini kit (Qiagen). A 45 cycle one-step qRT-PCR with annealing temperature of 57°C was carried out following the Luna Probe One-Step RT-qPCR kit (NEB) protocol. All available tissue samples from PhoPeV NGS-positive harbor porpoises were analysed using the newly developed qRT-PCR. An additional 109 kidneys from wild harbor porpoises that had stranded dead or alive along the Dutch North Sea coast and when alive had been nursed in the Dutch rehabilitation centre SOS Dolfijn for variable periods of time before dying, were also screened using this methodology. Spleen and brain tissue samples (if available) were also included from animals in which the kidney was found to be Pho- PeV PCR-positive.

Cell culture and virus isolation

PK-15 cells were cultured in DMEM media supplemented with 10 % FBS and 1 % penicillin/ streptomycin. MDBK cells were cultured in advanced MEM media supplemented with 10 % FBS, 1 % penicillin/ streptomycin and 1 % GlutaMax. Before virus isolation attempts, cells were washed with warm media without FBS and diluted kidney homogenates of samples NS170385 and NS170386 were added to 90 % confluent cells and incubated at 37°C with 5 % CO2 for 1–1.5 h. Cells were then washed twice and incubated overnight in growth media with 1 % FBS. Media was changed the next day. Cells were blind passaged after 3–4

days. Supernatant and cells were taken for PhoPeV-specific qRT-PCR analyses after each new passage.

RESULTS

Identification of a novel pestivirus

Lung and brain samples from three harbor porpoises with encephalitis indicative of viral infection were selected for NGS. Data was first analysed using a metagenomics pipeline [191], the results of which indicated the presence of a virus with homology to BVDV at the protein level in two of the animals (Figure S1, Supplementary Information). Assembly of contigs from these reads resulted in the discovery of a 11,880 bp sequence of a novel pestivirus, tentatively named Phocoena pestivirus (PhoPeV). The 5′ end of the new virus was determined by RACE due to low coverage in this region. The complete PhoPeV genome sequence (Gen- Bank accession nos. MK910227-29) was corroborated with Sanger sequencing data based on primers designed from the NGS reads. The two newly generated full-length genomes from the harbor porpoises differ 97.6 % in their genome. Sequence alignment of known pestivirus species indicated that PhoPeV is most related to the porcine pestiviruses Bungowannah virus and LINDA virus with approximately 60 % homology at the amino acid level, and only about 27 % homology to the more divergent BatPeV and APPV. Phylogenetic analysis using maximum likelihood estimations showed that PhoPeV also clusters with Bungowannah and LINDA viruses, forming a monophyletic group distantly related to other typical pestiviruses (Figure 1).

Absence of putative Npro coding region from the PhoPeV genome

PhoPeV has a polyprotein size of 3762 aa, which makes it smaller than other pestiviruses by approximately 150 aa, but similar in size with BatPeV (3663 aa) and APPV (3635 aa). The untranslated regions (UTRs) were of similar size to those of other pestiviruses, with a 5′UTR of 382 bp and 3′UTR of 212 bp. The most striking feature of the PhoPeV genome was the absence of Npro sequences (Figure 2a), as the alignment with other pestivirus species showed a gap at the start of the polyprotein which normally encodes Npro, followed by presence of sequences homologous to C. All other putative structural and non-structural pestivirus proteins were identified. Cleavage sites were recognized based upon homology with Bungowannah virus. The cleavage sites used by NS3 were all conserved, having Leu at P1 and Ser/Ala at P1′ positions. Interestingly, it was also noted that PhoPeV sequences from the lungof animal NS170385 had a 180nt insertion at the start of NS2. The inserted sequence was homologous to a sequence encompassing the C-terminal region of NS5A and N-terminal region of NS5B (Figure 2b).

The two variant sequences (with and without the insertion) were detected in the brain of the same animal (NS170386), although the variant without insertion was predominant according to the Sanger sequencing results (Figure S2, Supplementary Information).

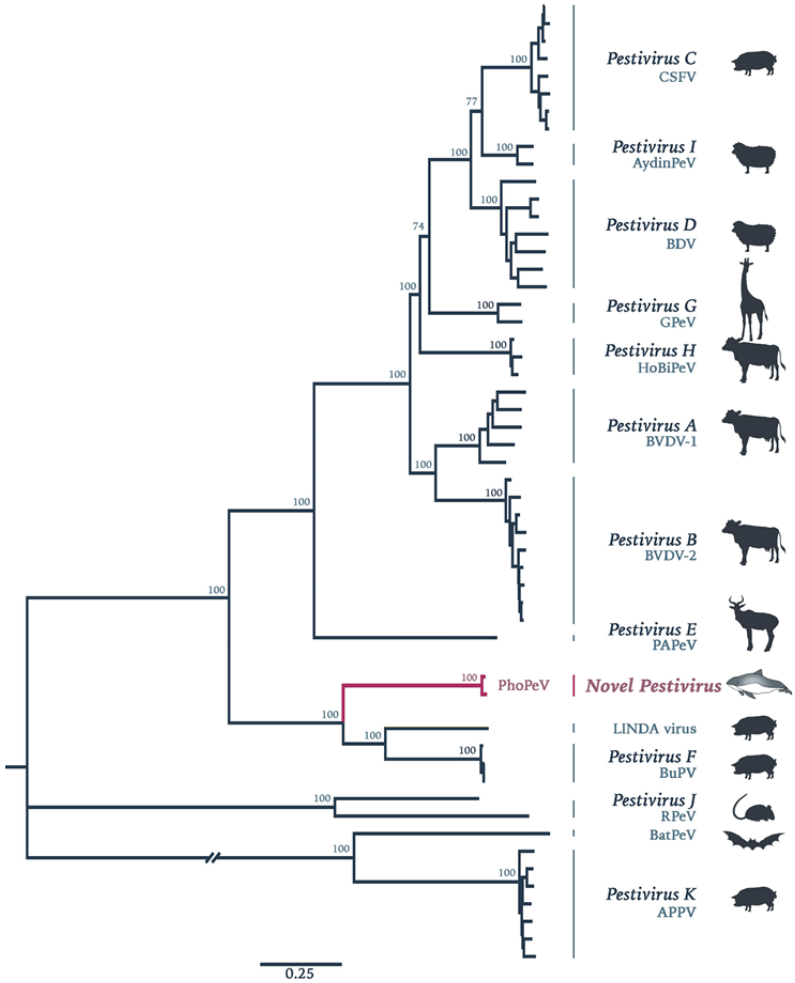

Figure 1. Phylogenetic reconstruction using maximum likelihood estimation of complete polyprotein of known and putative pestiviruses. Main bootstrap values are presented at nodes. Scale bar indicates number of amino acids changes per site. Taxon names are presented by the virus species and virus abbreviation. Abbreviations: CSFV, Classical swine fever virus; AydinPeV, Aydin-like pestivirus; BDV, Border disease virus; GPeV, Giraffe pestivirus; HoBiPeV, HoBi-like pestivirus; BVDV, Bovine viral diarrhea virus; PAPeV, Proghorn antelope pestivirus; PhoPeV, Phocoena pestivirus; LINDA, Lateral shaking inducing neurodegenerative agent; BuPV, Bungowannah porcine pestivirus; RPeV, Rat pestivirus; BatPeV, Bat pestivirus; APPV, Atypical porcine pestivirus of animal NS170385 had a 180nt insertion at the start of NS2. The inserted sequence was homologous to a sequence encompassing the C-terminal region of NS5A and N-terminal region of NS5B (Figure 2b). The two variant sequences (with and without the insertion) were detected in the brain of the same animal (NS170386), although the variant without insertion was predominant according to the Sanger sequencing results (Figure S2, Supplementary Information).

Pho-PeV tropism characterized by qRT-PCR and ISH

The two harbor porpoises positive by NGS for Pho- PeV had a systemic infection, as all analysed tissues were positive by both ISH (Table 1) and qRT-PCR (Table 2). All tissues of both porpoises expressed pestivirus RNA (Table 2). Pestivirus RNA was visible by ISH as red staining in the cytoplasm, and ranged from single granules to multiple granules to diffuse staining of the cytoplasm (Figure 3). Pestivirus RNA expression was seen neither in negative control samples nor in tissues of a porpoise that was negative for pestivirus by PCR. The cell types that expressed pestivirus RNA were mainly smooth muscle cells and epithelial cells, but a variety of other cell types were also involved (Table 1). Positive smooth muscle cells were found in the walls of small and medium-sized arteries of every tissue examined, as well as in the aorta wall. In addition, positive smooth muscle cells were found in the muscular layers of digestive tract tissues, urinary bladder, bronchi and bronchioles, and in the trabeculae

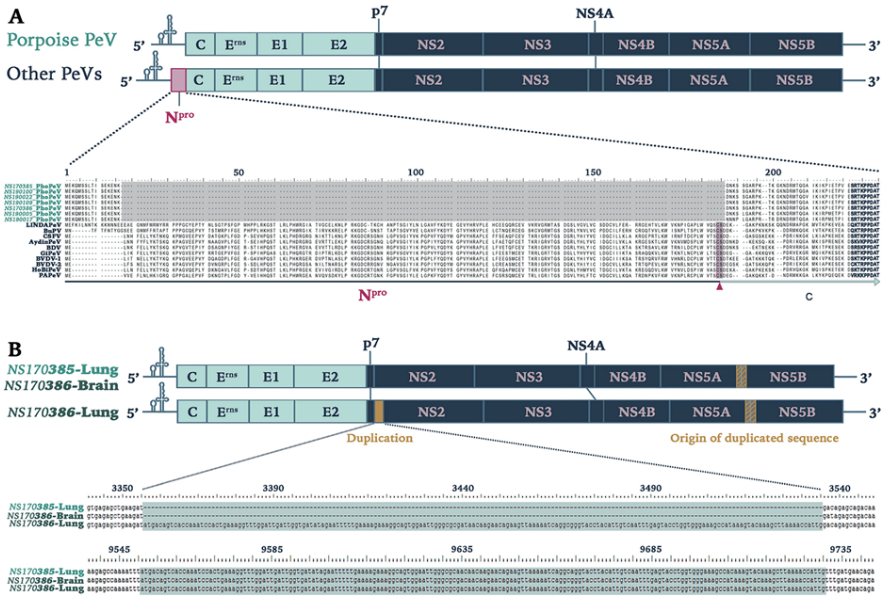

Figure 2. Genomic characterization of Phocoena pestivirus. (a) Comparison of PhoPeV to other pestivirus genome organization. PhoPeV genome arrangement is similar to other pestiviruses except for the unique absence of Npro in screened harbor porpoises (n = 7). Alignment of Npro area of PhoPeV and other pestivirus sequences is zoomed in. Absence of Npro gene coding sequences is highlighted in grey, conserved area in the capsid (from 221nt) is in bold. C/S cleavage site between Npro and Capsid is highlighted in pink and indicated with an arrowhead. Virus (Abbreviation – GenBank Accession No.): LINDA pestivirus (LINDAPeV-KY436034), Bungowannah pestivirus (BuPV-EF100713), Classical swine fever (CSFV-X87939), Aydin-like pestivirus (AydinPeV-JX428945), Border disease virus (BDV-AF037405), Giraffe pestivirus (GiPeV-AD144617), Bovine viral diarrhea virus-1

(BVDV-1-M31182), Bovine viral diarrhea virus-2 (BVDV-2-U18059), HoBi-like pestivirus (HoBiPeV-AB871953), Proghorn pestivirus (PAPeV-AY781152). (b) Comparison between PhoPeV sequences in different organs generated from two animals. The lung of animal NS170386 has an insertion (yellow) between p7 and NS2, which sequences originates from a region between NS5A and NS5B (striped yellow square). Duplicated and origin of duplicated sequence are zoomed in and are highlighted in teal color.

of the spleen. Positive epithelial cells were found in the mucosal lining of the respiratory, digestive, and urogenital systems. Specialized epithelial cell types expressing pestivirus RNA were keratinocytes, pancreatic acinar cells, bile duct epithelial cells, renal tubular epithelial cells, Sertoli cells, and thyroid follicle epithelial cells. Other cell types besides smooth muscle cells and epithelial cells also expressed PhoPeV RNA. These were neurons in the brain, cardiomyocytes in the heart, endocrine cells in the islets of Langerhans and adrenal gland, and mononuclear cells (tentatively identified as dendritic cells, lymphocytes, or both) in lymph nodes and spleen. Occasional mononuclear cells in hepatic sinusoids expressed pestivirus RNA and were tentatively identified as Kupffer cells.
Comparison of sequential tissue sections stained either by haematoxylin and eosin (for histopathological analysis) or by ISH (for PhoPeV pestivirus RNA expression) did not show any evidence of histological lesions caused by PhoPeV infection (Figure 3). Specifically, cells that expressed pestivirus RNA did not show evidence of cellular damage, and positive cells did not co-localize with histological evidence of inflammation, hemorrhage, or necrosis.

PhoPeV isolation in cell culture

Isolation of PhoPeV was performed with kidney homogenates of the two NGS-positive animals with the cell lines MDBK (bovine) and PK-15 (porcine). Virus replication was confirmed by qRT-PCR and NGS of PhoPeV isolate (NS170385k). Virus isolations were assessed by detection of PhoPeV viral load using qRT-PCR as the virus did not appear to generate cytopathic changes. After three passages in MDBK cells, Ct values of supernatant from cells infected with PhoPeV/ NS170386k were reduced from 33 to 22, indicating virus replication. In contrast, the amount of virus present in the supernatant of MDBK cells infected with PhoPeV/NS170385k homogenate remained in the same range throughout the three passages (Ct of 26). Similarly, after two passages in PK-15 cells, Ct values in supernatant dropped from 25 to 21 for PhoPeV/ NS170386k-infected cells, whereas Ct values in supernatant were maintained at approximately 22 for PhoPeV/NS170385k-infected cells. Sequencing of supernatant and infected PK-15 cells with PhoPeV/ NS170385 P2 by NGS confirmed the presence of PhoPeV.

Table 1. Cell types throughout the organ systems of two harbor porpoises (NS170385-86) infected by PhoPeV. Tissue sections were stained for PhoPeV RNA by *in situ* hybridization.

Organ system	Tissue	Cell types expressing pestivirus RNA
Nervous	Cerebrum	Neuron
	Cerebellum	Neuron
Cardiovascular	Heart ventricle	Cardiomyocyte
	Aortaa	Smooth muscle cell
	Artery	Smooth muscle cell
Respiratory	Blowhole[a]	Keratinocyte
	Trachea	Respiratory epithelial cell, submucosal gland epithelial cell
	Bronchus	Respiratory epithelial cell, submucosal gland epithelial cell, smooth muscle cell
	Lung	Bronchiolar smooth muscle cell, alveolar wall interstitial cell Digestive
Digestive	Lip[b]	Keratinocyte
	Esophagus	Surface epithelial cell
	Pharynxa	Surface epithelial cell
	Stomach	Gastric pit epithelial cell, smooth muscle cell
	Intestine	Enterocyte, smooth muscle cell
	Pancreas	Exocrine acinar cell, islet of Langerhans cell
	Liver	Bile duct epithelial cell, Kupffe
Urogenital	Kidney	Glomerular cell, tubular epithelial cell, collecting duct epithelial cell, pelvic epithelial cell
	Urinary bladder	Transitional epithelial cell, smooth muscle cell
	Uterusa	Surface epithelial cell
	Testis[b]	Sertoli cell, epididymal duct epithelial cell
Lymphoid	Spleen	Mononuclear cell, smooth muscle cell
	Lung-associated lymph node	Mononuclear cell Mesenteric lymph node
Endocrine	Adrenal gland	Cortical cell, medullary cell
	Thyroid gland	Follicle epithelial cell
Integumentary	Skin	Keratinocyte
Musculoskeletal	Skeletal muscle[b]	Negative

Table 2. Lesions and levels of PhoPeV RNA (inversely correlated with Ct values) in the tissues of PhoPeV-positive harbor porpoises.

Host_ID	Date of stranding	Days in rehabilitation	Lesions (gross pathology/histology)	Sample material	RT-PCR (Ct)
NS170385*	May 2012	9	COD: bronchopneumonia associated with nematode infection, cerebrum polioencephalitis multifocal mild, hepatitis necrotizing multifocal acute marked. Incidental lesions: adrenalitis with eosinphilic intranuclear inclusions lip ulcus, blowhole ulcer, pyloric stomach focal gastritis	Lung Brain Liver Kidney Spleen Bladder Muscle	21.1 26.5 27.7 15.9 20.2 19.2 19.3
NS170386*	Dec 2008	12	COD: bronchopneumonia associated with parasitic and bacterial infection, marked emaciation. Incidental lesions: epiglottal ulcer, necrotizing pharyngitis, necrotizing ulcerative esophagitis, cholangitis ulcerative dermatitis.	Lung Brain Liver Kidney Spleen Bladder Muscle Skin	18.2 22.6 19.6 16.4 18.8 17.6 21.1 25.7
NS190005	Mar 2003	26	COD: bilateral keratoconjunctivitis, pneumonia associated wiht bacterial infection. Incidental lesions: pneumonia associated dermatitis	Kidney Spleen Brain	32.5 37.3 38.2
NS190017	Apr 2004	35	COD: bronchopneumonia associated wiht lungworm larvae, pneumonia associated with Aspergillus infection. Incidental lesions: ulcura on gential slit, rostral tip of palatum durum and on cornea of left eye, colonic crypt abcesses	Kidney Spleen Brain	22.4 25.8 24.1
NS190022	Jun 2001	10	COD: pneumonia associated with a bacterial infection. Incidental lesions: oesophageal ulcerations, parasitic infections of stomach and pulmonary artery pneumonia associated lesion lymphadenopathy, pleuritis and pericarditis	Kidney Spleen	17.3 19.0
NS190025	Jul 2001	0#	cerebellar haemorhage focal, oesophageal ulcertion multifocal, conjunctivitis, catarrhal mild, dermatitis	Kidney Spleen Brain	37.0 Neg 38.0
NS190026	Apr 2001	0#	advanced state of autolysis, no abnormalities detected	Kidney Spleen	34.5 36.7

Host_ID	Date of stranding	Days in rehabilitation	Lesions (gross pathology/histology)	Sample material	RT-PCR (Ct)
NS190075	Jan 1998	13 years 8 months+	COD: pneumonia, hyperplasia of the papilla vater causing obstruction of the pancreatic duct	Kidney Spleen Brain	34.0 Neg 34.8
NS190100	Sep 2014	0#	pneumonia and dermatitis	Kidney Spleen Brain	20.8 30.7 30.8
NS190109	Jul 2005	8	COD: encephalitis associated with herpesvirus infection, Bronchopneumonia associated with nematode infection. Incidental lesions: pulmonary arteritis associated with nematode infection	Kidney Spleen	17.1 23.0

Notes: COD: cause of death; Ct: cycle threshold.
*Samples were also analyzed by NGS.
#Dead stranded.
+Kept in zoo collection.

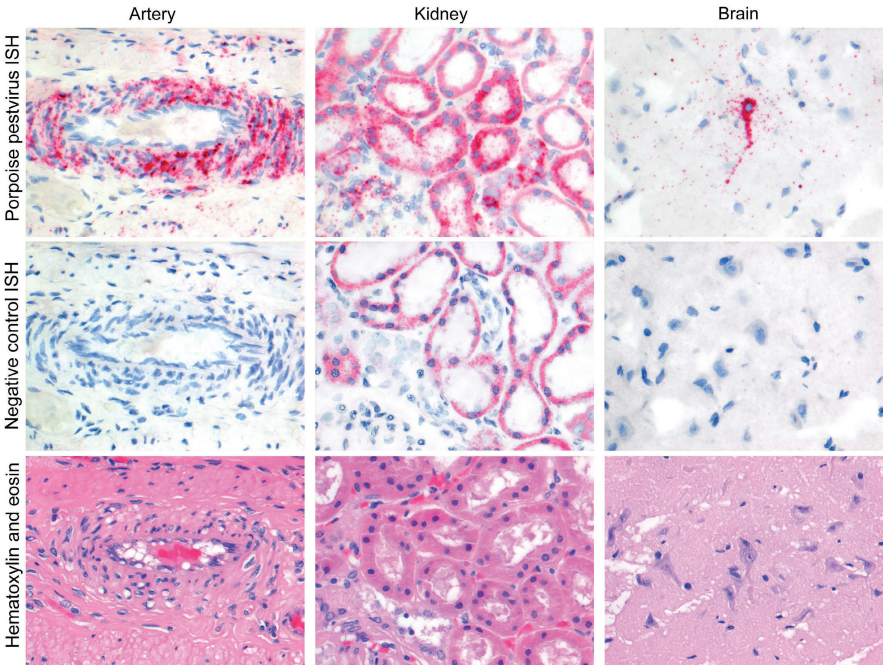

Figure 3. Phocoena pestivirus infects different cell types of harbor porpoises without histopathological changes. PhoPeV RNA expression is visible as bright red cytoplasmic staining in smooth muscle cells in the wall of an intestinal artery, epithelial cells in cortical tubules of the kidney, and neurons in the cerebrum of the brain, based on in situ hybridization (ISH) specific for Phocoena pestivirus (top row). Negative control ISH sections of these stain negative (middle row). Serial sections of these tissues, stained by hematoxylin and eosin, do not show any histopathological changes (bottow row). The narrow clefts in the neuropil of the brain are due to freeze-thaw artifact. Original objective magnifications for all panels: 40×. Artery and kidney were from porpoise NS170386, brain was from porpoise NS170385.

Prevalence and tissue distribution of PhoPeV among stranded North Sea harbor porpoises

Retrospective screening by qRT-PCR of stranded harbor porpoises along the Dutch North Sea coast in the period 2001–2014 showed a total of 10 out of 112 (9 %) animals positive for PhoPeV infection (Table 2). All other animals, which had died in the rehabilitation centre, including the ones that had been in direct contact with the positive animals, tested negative for pestivirus RNA in their tissues. Sequences between 5'UTR and C confirmed absence of Npro sequences in 7 out of 10 samples (Figure 2a). Three of these samples (NS190025-27) had relatively high Ct values between 34 and 37, therefore absence/presence of Npro sequences could not be corroborated. Analysis of concatenated 5'UTR, C, Erns, and E2 protein sequences (GenBank accession nos. MK910230-37) of a number of

PhoPeV PCR-positive animals indicated that different strains and two probable genotypes were present among harbor porpoises from the North Sea in this time period (Figure 4). Pairwise sequence identity between strain NS170017 and other PhoPeV strains showed 10 % difference at the nucleotide level.

DISCUSSION

Pestiviruses remain a major economic burden to livestock industry, due to incomplete control of classical pestiviruses such as CSFV, BVDV and BDV in many regions of the world. In the present study, we report the identification and characterization of PhoPeV, a novel evolutionary divergent pestivirus that lacks Npro and may cause a systemic infection in the harbor porpoise. In addition we show that 9 % (10/112) of stranded harbor porpoises from the Dutch North Sea coast carried PhoPeV.

Although most pestiviruses have been found in artiodactyl species, and cetacean species such as porpoises are considered to have evolved from them, the phylogenetic relatedness to other pestiviruses would not support the emergence of PhoPeV through co- evolution with the respective host species, but rather an interspecies transmission in the not too distant past. Moreover, the evolutionary relationship of PhoPeV, Bungowannah and LINDA viruses remains compelling, as these other viruses have only been found in pigs from Australia and Austria, respectively. It remains to be determined whether there was virus transmission between pigs and harbor porpoises at sometime in the past, or whether an intermediary host species has played a role in mediating transmission of the common ancestor of this pestivirus clade.

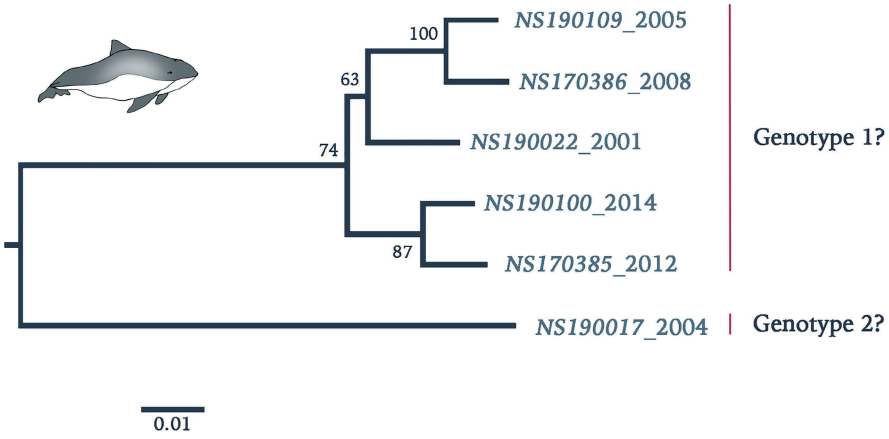

Figure 4. Phylogenetic reconstruction using maximum likelihood estimation of partial PhoPeV nucleotide genomes. Two main clades are highlighted as probable genotype 1 and 2. The PhoPeV nucleotide genomes used for analysis were 5'UTR, C, Erns, and E2 (GenBank accession nos. MK910230-37). Bootstrap values are presented at nodes. Scale bar indicates number of nucleotide changes per site LINDA virus was used as outgroup (GenBank accession number KY436034).

The identification of PhoPeV in two out of three porpoises with encephalitis appears to be coincidental rather than evidence of a causative role, as the virus was present in multiple cells of nearly all organs investigated with no preferential co-localization of viral RNA with brain lesions. Moreover, PCR tests for morbillivirus and herpesvirus, which have been previously associated with encephalitis [155, 187], were negative (data not shown). Therefore, the aetiology of the co-incidental encephalitis remained elusive. Consequently, tissue tropism and lack of PhoPeV associated lesions in the two positive porpoises investigated, most closely corresponds with that of ncp pestivirus infections, with BVDV infection in persistently infected cattle as the most striking parallel. It was previously reported that all 38 tissues from lymphoid, digestive, respiratory, endocrine, urogenital, nervous, cardiovascular, hematopoietic, and integumentary systems of two calves with persistent BVDV infection expressed virus antigen by immunohistochemistry [185, 186, 196-198]. Positive cells included lymphocytes, dendritic-like cells, macrophages, epithelial cells, and muscle cells. However, in common with our study, presence of viral RNA in tissues was not associated with tissue lesions [196]. We did not observe virus-associated encephalitis or glomerulonephritis, as have been diagnosed in some clinically healthy cattle persistently infected with BVDV [199]. In cattle, intrauterine infection of the foetus with ncp BVDV may induce selective immunotolerance, resulting in persistent viral infection in the absence of an adaptive immune response. Such animals can shed virus throughout their lifetime and are considered an important source of infection in the population [200, 201] It is interesting to note that like in ruminants, the placenta barrier of cetacean species is a complete epitheliochorial one [202], that may create similar conditions favouring intrauterine infection of the foetus. We speculate that a similar mechanism could explain the widespread PhoPeV infection and lack of associated pathological changes in the two investigated porpoises. Animals with analogous PhoPeV infections may represent an important source of virus transmission to naïve porpoises in the wider population. Nevertheless, given that these results are based on two animals and only one PhoPeV genotype, it remains to be determined whether this represented a true persistent infection or rather a transient infection, and whether PhoPeV infection of naïve porpoises, like in BVDV in cattle, is associated with enhanced pathology. Answers to these questions would have major consequences for health status of the population at large.

The most intriguing aspect of PhoPeV is the absence of the Npro gene. This protein acts as an antagonist of IRF3 and IRF7 in other pestiviruses [185, 186] and is not considered essential for viral replication in cell culture [197, 198], as its replacement with a murine ubiquitin gene in a recombinant CSFV strain did not affect viral replication in SK-6 cells. However, when this recombinant virus was used to infect pigs, no disease symptoms were displayed while high antibody titres were later detected [203]. In addition, it has been shown that complete deletion of Npro greatly compromises the growth rate of recombinant BVDV [204]. However, sequences directly downstream of the translation initiation codon have been suggested to be essential to pestivirus viability [205].

Therefore, in a recent study, the first four aa of Npro were retained upstream of the capsid gene, instead of a complete deletion of the Npro gene. This resulted in a slight reduction in the viral growth rate without compromising viability of the virus [206]. In the same study, recombinant strains of BVDV with either single or the combined mutations (almost complete deletion of Npro and/or deletion of codon 349 that abrogates Erns RNase activity) were used in pregnant cattle to evaluate their role in pestivirus persistent infection. Results indicated that only pregnant cattle infected with the double mutant strain cleared the infection, whereas virus reached foetuses and caused infection when inoculated with either wildtype or single mutant strains [206]. The ability of intact Erns or another PhoPeV protein to antagonize the porpoise innate immune system and thus mediate systemic virus spread remains to be determined. Alternatively, unique features of the cetacean immune system may have supported a loss of Npro from the ancestral PhoPeV. A loss of the Mx1 and Mx2 genes has been reported in toothed whales [207], the suborder to which the harbor porpoise belongs. Mx genes are important antiviral proteins, expression of which is regulated by type I interferon system, which in turn is controlled by IRF3 and IRF7 [208]. It remains to be elucidated whether the loss of Npro from PhoPeV is associated with differences in the innate immune response of cetaceans, such as the absence of Mx genes or any other interferon-stimulated gene.

The identification of PhoPeV as a novel putative member of the genus Pestivirus in a cetacean species expands the host range potential of pestiviruses, and clearly warrants further studies into the susceptibility of other cetacean species to PhoPeV or other related pestiviruses. The observed natural deletion of Npro from the PhoPeV genome highlights the genetic plasticity of pestiviruses and suggests that at least in one marine mammal species, systemic spread of a pestivirus is not predicated on the expression of Npro. Further research into the cetacean immune system and the role of Npro during pestivirus infections will help dissect mechanisms underlying the evolution and pathogenesis of this unique virus.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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Data availability
The sequences generated in this study from two full- length PhoPeV genomes have been deposited under GenBank accession numbers (MK910227-37). Other data are available upon request.

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harbour porpoise, Danish coastal waters
Jakob Højer Kirstensen

4

GENITAL DISEASE AND CENTRAL NERVOUS SYSTEM DISEASE IN HARBOR PORPOISES *(PHOCOENA PHOCOENA)* ARE ASSOCIATED WITH DIFFERENT HERPESVIRUSES

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ABSTRACT

Herpesvirus infection causes disease of variable severity in many species, including cetaceans. However, little is known about herpesvirus infection in harbor porpoises (*Phocoena phocoena*), despite being widespread in temperate coastal waters of the Northern Hemisphere. Therefore, we examined porpoises that stranded alive in the Netherlands, Belgium, and Germany between 2000 and 2014 for herpesvirus infection and associated disease. Porpoises that died or had to be euthanized were autopsied, and samples were collected for virological and pathological analyses. We found one known herpesvirus (*Phocoena phocoena* herpesvirus type 1, PPHV-1)—a gammaherpesvirus—and two novel herpesviruses (PPHV-2 and PPHV-3)—both alphaherpesviruses—in these porpoises. A genital plaque, in which PPHV-1 was detected, occurred in 1 % (1/117) of porpoises. The plaque was characterized by epithelial hyperplasia and intranuclear inclusion bodies that contained herpesvirus-like particles, and that stained positive by a PPHV-1-specific in situ hybridization test. PPHV-2 occurred in the brain of 2 % (1/74) of porpoises. This infection was associated with lymphocytic encephalitis, characterized by neuronal necrosis and intranuclear inclusion bodies containing herpesvirus-like particles. PPHV-3 had a prevalence of 5 % (4/74) in brain tissue, 5 % (2/43) in blowhole swabs, and 2 % (1/43) in genital swabs, but was not associated with disease. Phylogenetically, PPHV-1 was identical to a previously reported herpesvirus from a harbor porpoise, PPHV-2 showed closest identity with two herpesviruses fromd olphins, and PPHV-3 showed closest identity with a cervid herpesvirus. In conclusion, harbor porpoises may be infected with at least three different herpesviruses, one of which can cause clinically severe neurological disease.

INTRODUCTION

Herpesvirus infections can cause disease of variable severity in many species, including cetaceans [209]. The most common small cetacean species in the North Sea is the harbor porpoise (*Phocoena phocoena*) [210, 211]. This population of harbor porpoises is still vulnerable, according to IUCN criteria [212]. Therefore, monitoring of and research into morbidity and mortality factors is important for the conservation of the population. Specifically, trends in both prevalence and severity of herpesvirus infection potentially may provide information on changes in immune status of the harbor porpoise population, as these are known to increase when immune competence decreases [213, 214].

Mammalian herpesviruses belong to the ancient virus family *Herpesviridae* of the order *Herpesvirales*, which is subdivided into *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae* [55]. During cospeciation for millions of years, herpesviruses have adapted to their hosts resulting in a large diversity of herpesviruses with a restricted host range. Most mammalian species have at least one and frequently multiple herpesviruses, if they are looked for. This includes cetaceans, in several species of which herpesvirus infections have been documented. Herpesvirus-associated lesions in cetaceans range from mucosal and/or cutaneous lesions [56-58] to encephalitis [59-61] and disseminated systemic disease [46]. Mucosal lesions have been associated with *Gammaherpesvirinae* [57], encephalitis and disseminated systemic disease have been associated with *Alphaherpesvirinae* [46, 59, 60], and cutaneous lesions have been associated with *Alphaherpesvirinae* and *Gammaherpesvirinae* [57, 58]. Despite the fact that the harbor porpoise is a relatively common cetacean species and many stranded individuals in several countries have been autopsied [22-24], the only reported herpesvirus infections are a case of encephalitis associated with herpesviral antigen expression in affected neurons [60], and several cases of dermatitis, in which a gammaherpesvirus, tentatively named Phocoenid herpesvirus-1, was detected by PCR [58].

As part of a long-term study of morbidity and mortality factors of live-stranded harbor porpoises in the Netherlands, we here describe two cases of herpesvirus infection: a gammaherpesvirus (tentatively named *Phocoena phocoena* herpesvirus 1, PPHV-1) infection associated with a genital mucosal plaque, and an alphaherpesvirus (tentatively named *Phocoena phocoena* herpesvirus 2, PPHV-2) infection associated with encephalitis. In addition, we document a distinct alphaherpesvirus (tentatively named *Phocoena phocoena* herpesvirus 3, PPHV-3) in brain tissue, pulmonary lymph node, blowhole and genital slit. The data suggest that PPHV-3 has a higher prevalence of infection in live-stranded harbor porpoises than PPHV-2, both in the brain (5 % [4/74] vs 2 % [1/74]) and genital slit (2 % [1/43] vs 0 % [0/43]), but is not associated with disease. PPHV-1 was only found in association with genital plaque, which had a prevalence of 1 % (1/117) in live-stranded harbor porpoises. These results indicate that herpesvirus infections in harbor porpoises are more common than previously known and may be associated with severe, potentially fatal disease.

MATERIALS AND METHODS

Rescue and rehabilitation of live-stranded cetaceans

Since 1967, small cetaceans—mainly harbor porpoises— that strand alive along the Dutch, Belgian and German coasts have been rehabilitated at the Dolfinarium Harderwijk (Harderwijk, The Netherlands) and subsequently released into the wild. Since 2004, this activity has been organized in the form of an independent foundation, SOS Dolfijn, which operates at the same site. SOS Dolfijn has two 50 m^3 pools with fresh water to which salt is added. In the first period of rehabilitation, animals are observed 24 h per day and standard parameters are recorded, including respiration rate, cramps, food intake and defecation. In addition, other potentially relevant observations are recorded, including swimming behavior and alertness. As an animal improves, the level of observation and care diminish to a minimum of 9 h per day.

Between 2009 and 2014, the genital slits, blowholes and oral cavities of rescued harbor porpoises were sampled upon admission by use of a dry cotton swab for virological diagnosis. Swabs were stored in virus transport medium [Hank's balanced salt solution supplemented with 0.5 % lactalbumin, 10 % glycerol, 200 U/mL penicillin, 200 µg/mL streptomycin, 100 U/mL polymyxin B sulfate, 250 µg/mL gentamycin, and 50 U/mL nystatin (ICN Pharmaceuticals)].

Admission and rehabilitation of live-stranded harbor porpoises at SOS Dolfijn was authorized by the government of the Netherlands (application number FF/75/2012/036). Samples used in the present study were collected from harbor porpoises for diagnostic purposes by qualified personnel of the SOS Dolphin Foundation under veterinary supervision. SOS Dolfijn provided permission to the Department of Viroscience, Erasmus Medical Center to use the samples for the present study. No samples were collected from animals for research purposes.

Autopsy and histology

In recent years, survival of rehabilitated harbor porpoises at SOS Dolfijn has been close to 50 %. Since 2000, those harbor porpoises that die or have to be euthanized, based on poor prognosis, have been autopsied at the Department of Viroscience (Erasmus MC, Rotterdam, The Netherlands) as part of a long-term program to understand morbidity and mortality factors of the North Sea harbor porpoise population.

Autopsies were performed according to a standard protocol [89]. The following tissues were sampled for histology: adrenal gland, bronchus, cerebellum, cerebrum, colon, duodenum, esophagus, forestomach, fundic stomach, gonads, heart, jejunum, kidney, liver, lung, mesenteric lymph node, muscle, pancreas, pulmonary lymph node, pyloric stomach, skin, spleen, thymus, thyroid, trachea, tracheobronchial lymph node, and urinary bladder. Tissue samples were fixed in 10 % neutral-buffered formalin, routinely processed,

and embedded in paraffin. The 3-µm-thick sections were mounted on glass slides and stained with hematoxylin and eosin (HE) for light microscopy.

Electron microscopy

Formalin-fixed, paraffin-embedded samples of brain tissue and genital mucosa were deparaffinized and embedded in epoxy resin. Thin sections were prepared, stained with 6 % saturated uranyl acetate and lead citrate, and examined with a Philips Morgagni 268D electron microscope (F.E.I., Brno, Czech Republic).

In-situ hybridization

For detection of gammaherpesvirus RNA, 5-µm-thick tissue sections were stained with a commercial in situ hybridization (ISH) technique as described previously [19]. The probe was designed by Advanced Cell Diagnostics (Hayward, CA, USA), based on the 1246 base pair (bp) herpesvirus-specific DNA polymerase fragment obtained from next-generation sequencing and subsequent Sanger sequencing of tissue samples from porpoise #6 (Table 1). For ISH, the RNAscope 2.0 FFPE Assay (Advanced Cell Diagnostics, Inc.) was used according to the instructions of the manufacturer. In short, sections were deparaffinized in xylene, dehydrated in ethanol and subsequently pretreated to allow access to target RNA. The probe was hybridized for 2 h at 40 °C, signal was amplified with six amplification steps and finally the signal was visualized with Fast Red. The section was counterstained with hematoxylin and mounted with Ecomount.

Herpesvirus polymerase chain reaction and sequencing

The following porpoise tissues were sampled for polymerase chain reaction (PCR) (Table 1): brain (combined samples of cerebrum and cerebellum), genital plaque (porpoise #6 only), kidney, mammary gland cyst (porpoise #6 only), liver, lung, skeletal muscle (porpoise #6 only) spleen and urinary bladder. Tissue samples were stored at −70 °C until use. Tissue samples were thawed and homogenized using a Fastprep24 tissue homogenizer (MP Biomedicals, Santa Ana, CA, USA). From genital swab samples, a 200 µL aliquot was taken. DNA was isolated from tissue homogenates and swab samples using the High Pure Viral nucleic Acid Kit (Roche, Almere, The Netherlands), following the protocol provided by the manufacturer. A nested herpesvirus PCR was performed as described previously [215]. In brief, two forward primers (HV-F1: 5′-GAYTTYGCNAGYYTNTAYCC-3′ and HV-F2: 5′-TCCTGGACAAGCAGARNYSGCNMTN
AA-3′) and one reverse primer (HV-R1: 5′-GTCTTG CTCACCAGNTCNACNCCYTT-3′) directed to the polymerase gene were used in the first PCR. An aliquot of 2 µL from the first PCR reaction was used for a nested PCR with one forward primer (HV-F3: 59-GTAACTCGGTGTAYGGNTTYACNGGNGT-39) and one reverse primer (HV-R2: 59-CACAGAGTCC GTRTCNCCRTANAT-39). Products of the PCR reactions were checked

by electrophoresis on a 2 % agarose gel for fragments of the correct size. Automated sequencing of PCR fragments was performed on an ABI 3130XL genetic analyzer with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), using the nested herpesvirus PCR primers. For identification of sequenced fragments, the BLAST option of the National Center for Biotechnology Information website was used.

Table 1 Clinical and pathological evidence of central nervous system disease and genital disease in live-stranded harbor porpoises in which herpesvirus was detected

Porpoise number	Pathology number	Age category	Gender	CNSdisease			Genital disease		Herpesvirus	Tentative virus type
				Nervous signs	Encephalitis	INIB[a]	Mucosal INIB plaque		PCR positive samples	
1	PP040324	Juvenile	Male	No	Yes	No	No	No	Brain	PPHV-3
2	PP050825.2	Juvenile	Female	Yes	Yes	Yes	No	No	Brain	PPHV-2
3	PP051021	Juvenile	Male	No	Yes	No	No	No	Brain,pulmonary lymph node	PPHV-3
4	PP030320	Juvenile	Male	No	No	No	No	No	Brain	PPHV-3
5	PP060327.4	Juvenile	Male	Yes[b]	No	No	No	No	Brain, cornea	PPHV-3
6	PP050502	Adult	Female	No	Yes	No	Yes	Yes	Skeletal muscle, urinary bladder, kidney, skin lesion	PPHV-1
7	PP140627	Neonate	Female	No	No	No	No	No	Blowhole swab, Genital swab	PPHV-3
8	N.a[d]	Juvenile	Male	No	N.a.	N.a.	No	N.a.	Blowhole swab	PPHV-3

[a] Intranuclear inclusion bodies.
[b] Forceful expiration, body tremor, cramp.
[c] This porpoise was swabbed on arrival at rehabilitation centre and subsequently autopsied.
[d] Not applicable.`

Phylogenetic analysis

Phylogenetic analysis was performed by creating an alignment using the ClustalW method in MEGA6 [216], based on a polymerase gene fragment of the detected herpesviruses. A maximum likelihood phylogram was constructed using the maximum likelihood method based on the Kimura 2-parameter model with 500 boot- strap replicates.

Next-generation sequencing and detection of herpesvirus sequences

Tissue samples of three harbor porpoises (brain from porpoise #1 and #2, and skeletal muscle from porpoise #6) were processed for sequence-independent DNA virus

screening as described previously [217, 218]. In brief, tissues samples were defrosted and homogenized using a Fastprep24 tissue homogenizer (MP Biomedicals) in Hank's balanced salt solution supplemented with 0.5 % lactalbumin, 10 % glycerol, 200 U/mL penicillin, 200 µg/mL streptomycin, 100 U/mL polymyxin B sulfate, 250 µg/mL gentamycin and 50 U/mL nystatin (ICN Pharmaceuticals, Laval, Quebec, Canada) and centrifuged briefly. Supernatants from homogenates were filtered and the samples were treated with Omnicleave Endonuclease (Epicentre Biotechnologies, Madison, WI, USA). Subsequently, viral nucleic acids were extracted using the High Pure Viral Nucleic Acid kit (Roche) according to the instructions of the manufacturer. Random amplification was performed and amplicons were processed for next- generation sequencing with a 454 GS Junior instrument (Roche). Reads were trimmed and assembled using de- novo assembly in CLC Genomics Workbench 5.5.1 (CLC Bio, Aarhus, Denmark) and analyzed by nucleotide and translated nucleotide BLAST searches. Sequences were classified based on the taxonomic origin of the best-hit sequence using MEGAN software. E-values of e^{-10} were used as the cut-off value of significant virus hits for BLASTn and BLASTx.

Sanger sequencing

Using the 454-sequencing reads obtained from porpoise #6, open reading frame 56 (ORF56)-specific primers were designed based on ORF56 Ovine herpesvirus 2 to obtain partially overlapping PCR amplicons using AmpliTaq Gold DNA polymerase (Roche). The overlapping PCR fragments were sequenced, resulting in a 1246 bp fragment of the ORF56 homologue of the herpesvirus from porpoise #6 (GenBank accession number KU200258).

Papillomavirus PCR

A papillomavirus-specific PCR was performed on the clitoral plaque sample from porpoise #6 using primers MY11/MY09 [24] and GP5+/GP6+ [25]. This PCR had
been successful in detecting DNA of two papillomaviruses from Burmeister's porpoise (*Phocoena spinnipinnis*) [219].

Virus culture

Ten percent homogenates of selected tissue samples that had been stored at −70 °C were inoculated on the following cell types: primary harbor porpoise kidney cells, Madin-Darby bovine kidney cells, Madin-Darby canine kidney cells, and Crandell feline kidney cells. Cultures were washed twice with Dulbecco's modified Eagle's medium supplemented with antibiotics and 10 % fetal calf serum and incubated at 37 °C. Cultures were checked daily for cytopathic changes for a maximum of 10 days. At least three passages were made before cultures were considered negative. At the end of each passage, all cultures were tested for the presence of herpesviral DNA by PCR as outlined above.

RESULTS

Alphaherpesvirus PPHV-2 infection associated with encephalitis

Porpoise #2 was a juvenile female harbor porpoise (body weight 19.4 kg, standard body length 110 cm at autopsy) found alive on 11 July 2005 at Middelskerk (Oostende, Belgium) (Table 1). During the first 5 days after admission at the rehabilitation centre, the porpoise had difficulty to coordinate the surfacing of the blowhole and inspiration, resulting in inadvertent inspiration of water and coughing. During the next 3 days, the porpoise showed exaggerated surfacing with the entire head out of the water, possibly to prevent the inspiration of water. Breathing frequency remained within normal reference ranges (<5/ min). Shivering or tremors were observed during the entire 8 days in rehabilitation. The porpoise was euthanized on 19 July 2005 because of poor prognosis.

The main pathological diagnosis was a lymphocytic encephalitis with neuronal necrosis and intranuclear inclusion bodies (INIB). No macroscopic changes were seen in the brain, but histologically in the cerebrum, there was a locally extensive area of increased density of nuclei in the gray matter. In this area, blood vessels had lymphocytic cuffs about three cells thick and randomly scattered neutrophils in the neuropil. In multiple neurons, the cytoplasm was eosinophilic and the nuclei had large amphophilic inclusion bodies and marginated chromatin (Figure 1). By electron microscopy, the nuclei of these neurons contained many round or hexagonal particles with a consistent diameter of approximately 90 nm (Figure 2). Most particles had a round, electron-dense core, whereas others were empty. Size, shape and location of these particles are consistent with herpesvirus nucleocapsids [27]. Other significant diagnoses in this porpoise were multifocal pyogranulomatous pneumonia associated with nematode infection (probably *Stenurus minor*), multifocal proliferative arteritis of the pulmonary arteries associated with nematode infection (probably *Pseudalius inflexus*), and diffuse pulmonary oedema associated with euthanasia. By PCR, herpesvirus DNA (PPHV-2) was detected in the brain sample of this porpoise. The viral DNA polymerase 201 bp amplicon showed strongest identity with an alphaherpesvirus of a bottlenose dolphin (*Tursiops truncatus*) from Germany (92 %) and a striped dolphin (*Stenella coeruleoalba*) that died during the dolphin morbillivirus outbreak in Spain in 2011 (87 %) (Figure 3). Other tissues from this porpoise (kidney, liver, lung, spleen and urinary bladder) tested negative for herpesvirus DNA by PCR. Virus culture from brain, lung, spleen, kidney and urinary bladder samples of this porpoise was attempted, but no virus was cultured.

Based on this case report, we retrospectively screened brain samples of live-stranded harbor porpoises that were autopsied between 2000 and 2014 ($n = 74$) for herpesvirus infection and encephalitis. The animals identified included 1 male abortion, nine male neonates, 25 male juveniles, seven male adults, six female neonates, 16 female juveniles and 11 female adults. Brain samples were tested for herpesvirus DNA by PCR and examined for histopathological changes by light microscopy. If brain tissues were found positive for

herpesvirus DNA, other frozen tissues from the same porpoise were also tested. We found four additional porpoises (#1, #3 to #5) with herpesvirus DNA in the brain (Table 1). Two of these porpoises also had herpesvirus DNA in extra-neurological tissues: pulmonary lymph node and cornea (Table 1). The sequences of the PCR products from all four porpoises were identical to each other, but distinct from PPHV-2. The virus (PPHV-3) showed closest identity (86 %) with cervid herpesvirus from a North American elk (*Cervus canadensis*) (Figure 3). Therefore, the overall prevalence of PPHV-2 and -3 infections in the brain of live-stranded porpoises that were autopsied was 1 % (1/74) and 5 % (4/74), respectively. Notably, PPHV-3 had a predeliction for juvenile male porpoises: 16 % (4/25).

We found six additional porpoises with histopathological changes in the brain: three juvenile males and an adult female with inflammation of cerebrum, one adult male with inflammation of cerebrum, cerebellum and cervical spinal cord, and one juvenile female with inflammation of cerebellum and meninges. In general, these changes consisted of increased cell density in neuropil (gliosis), aggregation of macrophages around neurons (satellitosis), and aggregation of mononuclear cells around blood vessels (perivascular cuffing). Brain tissues of two (#1, #3) of these six porpoises also were positive for herpesvirus DNA (PPHV-3, Table 1). However, no neurons with INIB typical of herpesvirus infection were found in brain samples of any of these six porpoises. Consequently, it was not possible to attribute these histopathological changes to herpesvirus infection. In one porpoise, the juvenile female, the inflammation was associated with a mixed bacterial and fungal infection (data not shown). Therefore, the overall prevalence of encephalitis in live-stranded porpoises that were autopsied was 9 % (7/74).

Gammaherpesvirus PPHV-1 infection associated with genital plaque

Porpoise #6 was an adult female harbor porpoise (body weight 58.9 kg, standard body length 161 cm at autopsy) found alive on 24 November 2003 at Noordwijk aan Zee (The Netherlands). After long-term rehabilation at SOS Dolfijn the porpoise developed kidney failure. This was most evident from blood serum values: urea increased from 18 to 30 mmol/L, creatinine from <27 to 81 µmol/L and sodium from 158.5 to 196.4 mmol/L. The porpoise was euthanized on 2 May 2005 because of poor prognosis.

At autopsy, there was a plaque on the mucosa of the clitoris. It consisted of a round yellow-white soft nodule (18 mm long, 10 mm wide and 2 mm high) with an irregular surface. By histology, this plaque consisted of moderately thickened epithelium, characterized by hyperplasia and folding. In the upper half of the epithelium, many epithelial cells had large, round to oval, amphophilic INIB surrounded by a clear halo. The superficial connective tissue subjacent to the epithelium contained more capillaries than normal (Figure 4). By electron microscopy, the nuclei of these epithelial cells contained herpesvirus-like particles with similar characteristics as those described above, except that they had a diameter of approximately 110 nm (Figure 5). Other significant diagnoses in this porpoise

were suppurative bacterial dermatitis and granulomatous pneumonia of unknown cause. By PCR, herpesvirus DNA (PPHV-1) was detected in kidney, mammary gland cyst, skeletal muscle and urinary bladder samples of this porpoise. The viral DNA polymerase 372 bp amplicon showed 100 % identity with a gammaherpesvirus detected in another harbor porpoise from the Netherlands [58] (Figure 3). Other tissues from porpoise #6 (including brain, clitoral plaque, liver, lung and spleen) all tested negative for herpesvirus DNA by PCR. Virus culture from clitoral plaque, vaginal nodule, mammary gland cyst, skeletal muscle, brain, kidney, lung, spleen and urinary bladder samples of porpoise #6 was attempted, but no virus was cultured.

By next-generation sequencing of the skeletal muscle sample, which had the strongest herpesvirus DNA polymerase PCR band of all PCR-positive tissues, multiple reads of herpesvirus were obtained and used to design specific primers and obtain multiple overlapping PCR fragments. This resulted in a 1246 bp fragment homologous to gammaherpesvirus ORF56. By ISH using a probe designed based on this fragment, gammaherpes- virus RNA was detected in epithelial cells of the clitoral plaque (Figure 4). Besides herpesvirus, this skeletal muscle sample also contained four reads of papillomavirus, ranging from 232 to 330 bp and spanning a total length of 1170 bp. By BLAST analysis, the identities ranged from 73 % with *Phocoena spinnipinnis* papillomavirus (accession number: AJ23837.1) for a 327 bp fragment to 83 % with *Miniopterus schreibersii* papillomavirus (GenBank accession number: JQ692938.1) for a 41 bp stretch of a 232 bp fragment. The clitoral plaque sample tested negative in the papillomavirus PCR.

Based on this case report, we prospectively screened harbor porpoises that stranded alive between 2009 and 2014 ($n = 43$) for herpesvirus infection and genital plaques. These porpoises were one male neonate, 18 male juveniles, two male adults, three female neonates, 16 female juveniles and three female adults. Swabs were collected from genital slits, blowholes and oral cavities and tested for herpesvirus DNA by PCR. Of these 43 porpoises, 16 were later autopsied. Furthermore, we screened autopsy reports of the live-stranded harbor porpoises that were autopsied between 2000 and 2014 ($n = 74$). We found two additional porpoises (#7 and #8) with swabs positive for herpesvirus DNA (Table 1). In one, genital swab and blowhole swab were positive; in the other, only the blowhole swab was positive. The sequences of the PCR products from both porpoises were identical. The virus (PPHV-3) showed closest identity (86 %) with cervid herpesvirus from a North American elk (*Cervus canadensis*) (Figure 3). No additional porpoises that were autopsied or that were swabbed at admission had evidence of genital plaques. Therefore, the overall prevalence of herpesvirus infection in the genital mucosa of live-stranded porpoises was 2 % (1/43), while the overall prevalence of genital plaques was 1 % (1/117).

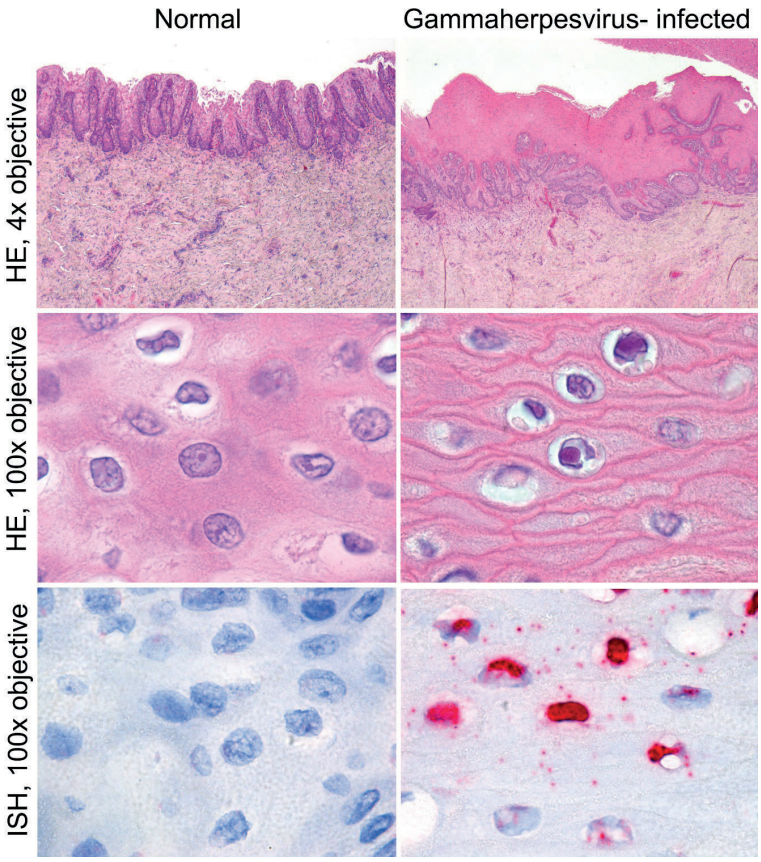

Figure 4 Genital plaque associated with *Phocoena phocoena* herpesvirus type 1 (PPHV-1) infection in a harbor porpoise. Genital mucosa of harbor porpoise #6 infected with PPHV-1, a gammaherpesvirus. Tissue sections are either stained with hematoxylin and eosin (HE) or by in situ hybridization (ISH) specific for RNA of PPHV-1. Left column shows normal genital mucosa and right column shows genital plaque infected with PPHV-1. The genital plaque is characterized by marked hyperplasia of the epithelial layer (top row). In the superficial part of this hyperplastic epithelium, many epithelial cells have nuclei with amphophilic intranuclear inclusion bodies, characteristic for herpesvirus infection (middle row). These nuclei express PPHV-2 RNA, visible as bright red staining (bottom row).

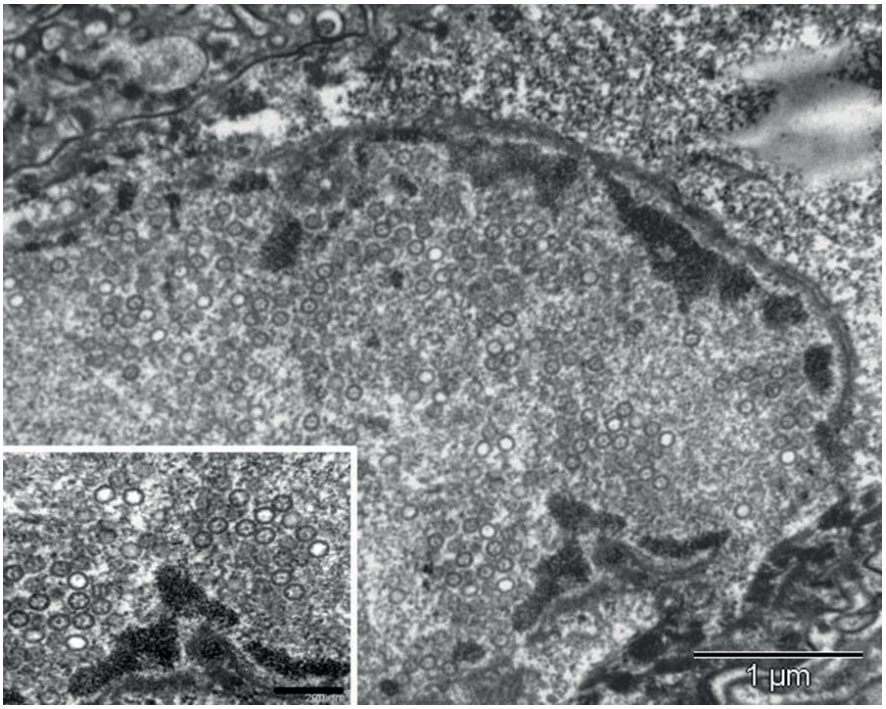

Figure 5 Herpesvirus-like particles in epithelial cell of a harbor porpoise with *Phocoena phocoena* herpesvirus type 1 infection.
Genital mucosa of harbor porpoise #6 infected with Phocoena phocoena herpesvirus type 1, a gammaherpesvirus. Transmission electron micrographs of an epithelial cell, stained with uranyl acetate and lead citrate. The nucleus of the epithelial cell has electron-dense clumps of marginated chromatin and contains an intranuclear inclusion body. Within this inclusion body, there are round to hexagonal unenveloped viral nucleocapsids, some with an electron-dense core (inset). Bar of inset = 200 nm

DISCUSSION

In this study, we demonstrated infection of live-stranded harbor porpoises with three different herpesviruses: PPHV-1, previously identified by van Beurden et al. [58], and the newly discovered PPHV-2 and PPHV-3. Of these three herpesviruses, the most important as a mortality factor for harbor porpoises was PPHV-2, which was associated with severe encephalitis in a juvenile female harbor porpoise (animal #2; Table 1). Evidence that PPHV-2 infection was the cause of encephalitis were: (1) detection of characteristic INIB in neurons of the affected brain tissue by light microscopy, (2) demonstration of herpesvirus-like particles within these INIB by electron microscopy and (3) detection of PPHV-2-specific DNA in brain samples of the affected porpoise by PCR and sequencing. The combination of

clinical observations, showing uncoordinated surfacing and respiration, and pathological analysis, showing severe PPHV-2-associated encephalitis, indicated that PPHV-2 infection of the brain was clinically significant and the most probable cause of stranding.
Encephalitis is commonly diagnosed in harbor porpoises, but its cause is rarely established. Encephalitis was a significant diagnosis in 9 % (7/74) of porpoises in this study, 3 % (4/133) of porpoises stranded along the German coast [23], and 11 % (6/55) of porpoises stranded along the Belgian coast [22]. One of the Belgian encephalitis cases was caused by *Streptococcus equisimilis* sepsis and two of the German encephalitis cases were due to ß-hemolytic streptococcal septicemia. In a harbor porpoise stranded in Sweden, Kennedy et al. [60] diagnosed herpesvirus as the cause of encephalitis, based on characteristic INIB in neurons and the demonstration of herpesvirus-like particles by electron microscopy. They suspected it was an alphaherpesvirus based on immunohistochemistry.

The only other cetacean species in which herpesvirus was associated with encephalitis are the bottlenose dolphin and the striped dolphin. Esperon et al. [59] detected herpesvirus DNA by PCR in the brain sample of a stranded bottlenose dolphin with a mild non-suppurative encephalitis. The DNA polymerase of the herpesvirus from the bottlenose dolphin had 98 % identity with that of herpes simplex virus 1. Sierra et al. [61] detected herpesvirus DNA by PCR in the brain of a striped dolphin with severe diffuse non-suppurative meningoencephalitis. The DNA polymerase of the herpesvirus from the striped dolphin was most closely related to herpesviruses observed in the lungs of a Cuvier's beaked whale (*Ziphius cavirostris*) and in another striped dolphin. PPHV-2, the striped dolphin herpesvirus and the bottlenose dolphin herpesvirus belong to the subfamily of *Alphaherpesvirinae*, suggesting that these viruses have a tropism for the nervous system and are potentially pathogenic in cetaceans. More extensive sampling of the brain to account for localized infection, and the application of molecular methods (e.g., PCR and next-generation sequencing) in conjunction with histological methods (e.g., immunohistochemistry and ISH) for pathogen detection are warranted to elucidate the causes of encephalitis, including herpesvirus infection, in harbor porpoises and other cetacean species.
The next herpesvirus we detected in these harbor porpoises, PPHV-1, was associated with a plaque in the clitoral mucosa of an adult female harbor porpoise (animal #6; Table 1). Evidence that PPHV-1 infection was the cause of this genital plaque were: (1) detection of characteristic INIB in epithelial cells of the affected mucosa by light microscopy, (2) demonstration of herpesvirus-like particles within these INIB by electron microscopy and (3) expression of PPHV-1-specific DNA in affected epithelial cells by ISH. The report of a similar plaque, containing herpesvirus-like particles by electron microscopy, in the penile mucosa of a harbor porpoise [220] indicates that the genital mucosa of both male and female harbor porpoises may be affected. In contrast to these genital plaques, van Beurden et al. [221] found PPHV-1 in association with skin lesions, characterized by

epidermal hyperplasia and INIB.

Genital lesions associated with herpesvirus infection have been found in four other cetacean species: a male Blainville's beaked whale (*Mesoplodon densirostris*) [222], a female Risso's dolphin (*Grampus griseus*) [57], a male striped dolphin [223] and both male and female bottlenose dolphins [57, 224, 225]. In the Blainville's beaked whale [222], the bottlenose dolphin [225] and the striped dolphin [223], the mucosal lesions were histologically similar to those reported in harbor porpoise #6: a well-demarcated area of epithelial hyperplasia, characterized by INIB in affected epithelial cells. In these four cetacean species, as well as in the harbor porpoise, the herpesvirus associated with these genital lesions belonged to the subfamily *Gammaherpesvirinae*. In a zoo collection of bottlenose dolphins, infection was endemic and seroconversion occurred around the age of onset of sexual behavior [225]. This epidemiological observation, together with the predilection for the genital mucosa, indicates that sexual contact is an important route of transmission of gamma- herpesviruses in cetaceans.

There is ongoing debate about the roles of herpesvirus infection and papillomavirus infection as the cause of genital lesions in cetaceans [226]. In that perspective, it is of interest that papillomavirus was detected in a skeletal muscle sample of harbor porpoise #6 by next-generation sequencing. Although we did not detect papillomavirus by PCR in the clitoral plaque sample of this animal, it cannot be excluded that the primers used—based on papillomaviruses from Burmeister's porpoise—are not suited to detection of papillomavirus of harbor porpoises. Therefore, the co-involvement of papillomavirus infection in the etiology of genital lesions in harbor porpoises needs to be explored further.

The last herpesvirus we detected in harbor porpoises, PPHV-3, was not related to disease. Despite detection in brain, pulmonary lymph node, genital slit and blowhole samples of several porpoises (animals #1, #3, #4, #5, #7 and #8; Table 1), no association with pathologic changes in the respective tissues was observed. It remains to be determined why PPHV-3 had an apparent predilection for juvenile male harbor porpoises and what the pathogenic potential of this virus is.

Because this research was part of a long-term study of morbidity and mortality factors in live-stranded harbor porpoises, we had a substantial sample size to evaluate the prevalence of both herpesvirus-associated encephalitis and genital plaques. The prevalence of PPHV-2-associated encephalitis was low (1 %; 1/74), indicating that this morbidity factor is rare in harbor porpoises that strand alive on the Dutch and adjacent coasts. However, a caveat is that only one brain sample (a sample of cerebral tissue and a sample of cerebellar tissue in one vial) was collected for virological analysis as part of our standard autopsy protocol. Given that herpesvirus infections may be limited to certain areas of the brain [227], we may have underestimated the number of porpoises with PPHV-2 in the brain. The low prevalence of herpesvirus-associated genital plaques in live-stranded harbor porpoises—0 % (0/52) in juvenile and adult males, 2 % (1/46) in juvenile and adult females—contrasts with the much higher prevalence in bottlenose dolphins in

a zoo collection—23 % (3/13) in juvenile and adult males, 27 % (4/15) in juvenile and adult females [225]. Similarly, regardless of the presence or absence of genital plaques, PPHV-1 infection was undetectable in the genital slit of harbor porpoises—0/20 for juvenile and adult males, 0/19 for juvenile and adult females—compared to a much higher prevalence of *Tursiops truncatus* herpesvirus type 1 infection in bottlenose dolphins in a zoo collection—19 % (4/21) for juvenile and adult males, 33 % (5/15) for juvenile and adult females [225]. Possible explanations are differences in herpesvirus, host species, or captive versus free-living populations.

To obtain a better idea of the prevalence of infection with these herpesviruses in autopsied harbor porpoises, it would be important to determine the site of latent infection and to sample those sites. For example, herpes simplex virus 1 in human beings establishes a latent infection in the root ganglia of the trigeminal nerve [228]. The development of serological assays to detect specific antibodies against PPHV-1, PPHV-2, and PPHV-3 would make it possible to estimate the prevalence of infection in live harbor porpoises. Such a serological assay, based on cultured virus, was successfully developed to detect specific antibodies against *Tursiops truncatus* herpesvirus in bottlenose dolphin sera [225].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CvE participated in the design of the study, carried out the autopsies and drafted the manuscript. MvdB carried out the in situ hybridization, polymerase chain reaction and phylogenetic analysis and assisted in drafting the manuscript. PvR carried out the immunohistochemistry and technical assistance for histology and assisted in drafting the manuscript. AdJ carried out the electron microscopy and assisted in drafting the manuscript. SG helped to develop the in situ hybridization test. GV provided expertise on herpesvirus. AO provided expertise on virology. TK conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Bottlenose dolphin, Lagune Dolfinarium Harderwijk, the Netherlands
Paulien Bunskoek

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GENITAL HERPESVIRUS IN BOTTLENOSE DOLPHINS *(TURSIOPS TRUNCATUS)*: CULTIVATION, EPIDEMIOLOGY AND ASSOCIATED PATHOLOGY

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ABSTRACT

We studied the pathology, epidemiology, and clinical significance of genital herpesvirus infection in a zoo collection of bottlenose dolphins (*Tursiops truncatus*). Samples from the genital mucosa of male (*n*=21) and female (*n*=15) dolphins were tested by nested polymerase chain reaction (PCR) targeting the DNA polymerase of herpesvirus. Herpesvirus infection was significantly associated with the occurrence of mucosal plaques on penis (*n*=3) or vulva (*n*=4). Biopsies from a penile plaque showed epithelial hyperplasia by histology, contained herpesvirus-like particles by electron microscopy, and tested positive for herpesvirus by PCR. Herpesvirus was successfully cultivated from penile plaque samples and identified as a member of the *Gammaherpesvirinae* by DNA sequencing and phylogenetic analysis. We used the newly cultivated bottlenose dolphin herpesvirus (TTHV) to develop a direct enzyme-linked immuno-sorbent assay for anti-TTHV antibodies in banked sera of these dolphins. The percentage of positive samples was higher in adults (20/21, 95 %) than in juveniles (7/15, 47 %). Seroconversion occurred around the age of onset of sexual behavior. Although herpesvirus infection has been associated with abortion, perinatal mortality, and urogenital neoplasia in other species, we found no evidence of herpesvirus infection by PCR in tissues from six cases of abortion and perinatal mortality, and no diagnoses of urogenital tumors in 24 bottlenose dolphins from this zoo collection that died since 1990. Together, we here report the first successful cultivation from bottlenose dolphins of a herpesvirus that probably causes benign genital plaques, is endemic in this group of dolphins, and is likely transmitted by sexual contact.

INTRODUCTION

Herpesviruses are widely disseminated in nature. Upon examination, most animal species yield at least one and frequently several distinct herpesviruses. Herpesviruses are well adapted to their natural hosts, and infections in immunocompetent individuals are rarely fatal [55]. Cetacean species have been found to be infected with herpesvirus by various methods. Associated lesions vary in location and severity. By phylogenetic analysis of the partial DNA polymerase coding sequence detected by polymerase chain reaction (PCR), these viruses belong to the alpha and gamma families of herpesviruses. Herpesvirus has never been cultivated from cetaceans. Skin lesions in beluga whales (*Delphinapterus leucas*)[37, 56] and bottlenose dolphins (*Tursiops truncatus*)[229] contained herpesvirus-like particles observed by electronmicroscopy. In addition, skin and oral lesions from bottlenose dolphins contained herpesviral DNA by PCR that was classified as belonging to an alphaherpesvirus [57]. Encephalitis in a harbor porpoise (*Phocoena phocoena*) was caused by herpesvirus infection as demonstrated by immunohistochemistry and electron microscopy [60]. A disseminated herpesvirus infection was observed in two bottlenose

dolphins with necrotizing lesions and eosinophilic intra- nuclear inclusion bodies in multiple organs. Lesions contained herpesvirus-like particles by electron microscopy and herpesvirus DNA by PCR belonging to the family of alphaherpesviruses [46]. Genital lesions associated with herpesvirus infection have been found in three cetacean species. A penis lesion in a Blainville's beaked whale (*Mesoplodon densirostris*) had basophilic intranuclear inclusion bodies and contained herpesviral DNA by PCR that was classified as belonging to a gammaherpesvirus [222]. Genital lesions from five bottlenose dolphins and one Risso's dolphin (*Grampus griseus*) contained herpesvirus DNA by PCR that were classified as belonging to gammaherpesviruses [57].

Besides benign proliferative lesions in the genital mucosa, genital herpesvirus infection is a known cause of severe clinical disease. Genital herpesvirus infections can occur in the gravid uterus and the neonate upon birth, and they may be dangerous because neither fetus nor neonate is immunocompetent [230]. Herpesvirus infection has been described as a cause of abortion, stillbirth, and neonatal disease in cattle, pigs, dogs, horses, and humans [230-232]. In California sea lions (*Zalophus californianus*), genital herpesvirus infection also has been implicated in the development of urogenital tumors, which are a significant cause of morbidity and mortality in this species [233].

Our goals in this study were first, to determine the etiology of lesions observed in the genital mucosa of bottlenose dolphins in a zoo collection; second, to investigate the epidemiology of the detected herpesvirus infection; and, third, to investigate whether infection with this herpesvirus could be linked to perinatal mortality or urogenital neoplasia.

MATERIALS AND METHODS

Bottlenose dolphins

The zoo collection of bottlenose dolphins under investigation consisted of 36 individuals. Based on sexual maturity at 10 yr of age [234], there were 15 females (11 adults and four juveniles) and 21 males (10 adults and 11 juveniles) (Table 1). This group consisted of wild-caught dolphins from the Gulf of Mexico (*n*=7), the waters around Cuba (*n*=1), and the Atlantic coast of Florida (*n*=1), as well as captive-bred dolphins (*n*=27). The dolphins were held in two marine mammal parks, one park in the Netherlands and the other park in France. The marine mammal park in the Netherlands consisted of two basins with volumes of 4 and 12 million liters and that in France of one basin with a volume of 4 million liters. These were filled with either fresh water supplemented with sodium chloride or with artificial seawater. Water was sand filtered and either chlorinated or disinfected using ultraviolet light. The dolphins were fed on different species of thawed frozen fish and squid, supplemented with vitamin B and E. All dolphins were under close daily supervision during training by experienced staff, and they were inspected at least once

per week by a registered veterinarian. Throughout the history of both parks, dolphins had been exchanged between the two parks and with other facilities for breeding purposes. At the time of this investigation, October 2007–October 2008, all dolphins from both parks were kept together in the Netherlands.

Samples of genital mucosa

From 34 dolphins, samples were collected in duplicate from the genital slit (which is present in both females and males) by use of a cotton swab. One cotton swab was placed in a vial with 1 ml of virus transport medium (Hanks' balanced salt solution [Hanks' minimal essential medium, HMEM] containing 10 % glycerol, 200 international units/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, and 250 µg/ml gentamicin) for virus culture and stored at room temperature until processing. The other cot- ton swab was placed in a vial with 0.5 ml of lysis buffer (High Pure Viral Nucleic Acid Kit, Roche Diagnostics GmbH, Mannheim, Germany) for PCR and stored at room temperature until processing. Two males with visible lesions in the penis mucosa (5 and 34) were sampled in duplicate by scraping a plastic bacteriology loop across the lesion. The plastic loops were shaken in vials containing either HMEM for virus culture or lysis buffer for PCR. One male (19) developed a penile lesion during the study period and was sampled on three occasions: first, by use of cotton swabs; second (after the penile lesion was detected), by use of plastic loops; and third, by use of disposable pulmonary biopsy forceps oval cup, outer diameter 1.8 mm (ConMed Endoscopy Technologies Inc., Billerica, Massachusetts, USA). On the third occasion, three tissue samples were taken at the edge of the lesion, two samples for histology, and one sample for PCR.

Table 1. Detection of herpesvirus infection and associated plaques in genital mucosa of bottlenose dolphins by PCR and macroscopic inspection, as well as serologic evidence of herpesvirus infection based on ELISA in oldest and most recent available samples from serum bank.

						Herpes ELISA				
	Dolphin			Genital mucosa[b]		Age at sampling(yr)		Titer[c]		Seroconversion
Age category and gender	No.	Origin[a]	Year of birth	Herpes PCR	Gross lesion	1st sample	2nd sample	1st sample	2nd sample	Between ages
Adult female	1	WC	1962 (est.)	N	N	33	46	>160	>160	
	8	WC	1967 (est.)	P	P	27	41	>160	>160	
	31	WC	1973 (est.)	N	N		35		>160	
	16	WC	1977 (est.)	P	P	25	31	>160	>160	
	3	WC	1982 (est.)	N	N	12	26	>160	>160	
	2	WC	1983 (est.)	P	P	13	25	N	>160	13-14
	15	CB	1987	N	N	11	21	>160	>160	
	13	CB	1989	N	N	8	19	>160	>160	
	10	CB	1992	N	N	6	16	>160	>160	
	9	CB	1992	N	N	10	16	>160	>160	
	35	CB	1996	P	N		12		N	
Juv. female	36	CB	1999	P	P		9		N	
	21	CB	2001	N	N	3	7	N	>160	4-5
	29	CB	2003	N	N	4	5	N	N	
	27	CB	2005	N	N	1	3	N	N	
Adult male	6	WC	1963 (est.)	N	N	31	45	>160	>160	
	5	WC	1974 (est.)	P	P	20	34	>160	>160	
	12	WC	1983 (est.)	N	N	15	25	>160	>160	
	34	WC	1988 (est.)	P	P	19	20	>160	>160	
	11	CB	1981	N	N	17	27	>160	>160	
	14	CB	1984	N	ne	6	24	40	>160	
	4	CB	1984	N	N	9	24	80	>160	
	7	CB	1989	N	N	6	19	N	>160	6-9
	17	CB	1998	N	ne	4	10	>160	>160	
	18	CB	1998	N	N	9	10	>160	>160	
Juv. male	32	CB	1999	N	N		9		N	
	19	CB	2001	P[d]	P[e]	1	7	N	N	
	20	CB	2001	N	ne	3	7	N	>160	3-4
	22	CB	2001	P	ne	1	7	N	>160	
	23	CB	2002	N	N	2	6	>160	>160	
	24	CB	2004	N	ne	2	4	N	>160	2-3
	30	CB	2004	N	N		4		N	
	33	CB	2004	N	N		4		N	
	25	CB	2005	N	ne	1	3	N	>160	2-3
	26	CB	2005	N	ne	1	3	>160	>160	
	28	CB	2005	N	ne		3		N	

[a] WC, wild-caught; CB, captive-born. [b] P, positive; N, negative; ne, not examined. [c] Value is dilution at which sample signal still more than twice control signal; N, sample signal less than twice control signal. [d] Negative in March 2008, but positive in October 2008, after penile plaque had developed. [e] No gross lesion visible before 8 September 2008, but visible on 25 September 2008.

Serum samples

Serum samples from each of the 36 dolphins had been collected as part of routine health evaluation. After collection of blood in a serum separation tube and clotting, the tubes were centrifuged at 20,000 3 G for 5 min, and obtained serum was archived at -20 °C.

Organ samples

Organ samples from four cases of perinatal mortality and two abortions were stored frozen at -70 °C until processing for herpesvirus PCR. These six cases were two aborted fetuses (3 and 6 mo old), two stillborn calves, one calf with a bilateral microphthalmia, and one calf that failed to thrive. Organs sampled were brain, lung, spleen (except from the 3-mo-old aborted fetus), liver (except from one stillborn calf), and placenta (except from the nonthriving calf). The 6-mo-old aborted fetus was severely damaged before it could be retrieved from the basin; thus, alternative samples were collected: placenta, heart, stomach, and adrenal gland.

Inspection of genital mucosa

The genital mucosa was inspected for the presence of lesions. In males, this was done during voluntary or spontaneous penis extrusion. In females, which all voluntarily presented the genital slit, the genital mucosa was exposed by spreading the genital slit by hand and shining a light onto the mucosa.

The frequency at which the genital mucosa was inspected during the study period varied per dolphin. In males, dolphins 12, 5, 6, 11, 7, 4, and 18, which were trained to extrude the penis voluntarily for semen collection, were inspected at least four times a month during the whole study period; dolphins 23 and 19 were inspected four times during September 2008; dolphin 34 was inspected twice during the study period; dolphins 32, 30, and 33 were inspected minimally once during spontaneous penis extrusion; and dolphins 14, 17, 20, 22, 24, 25, 26, and 28 were not examined. In females, the genital mucosa of each dolphin was inspected once during the study period.

Virus culture

Within 48 hr of sampling, 60 ml of virus transport medium that held the cotton swab of the genital mucosa (dolphins 16, 26, and 35) or the material scraped from a macroscopic male genital mucosal lesion (dolphins 34 and 5) was inoculated for 1 hr at 37 °C on each of the following cell lines: primary harbor porpoise kidney cell cultures (PPki), primary bottlenose dolphin kidney cell cultures (TTki), Madin- Darby bovine kidney cells (MDBK),

Madin- Darby canine kidney cells (MDCK), and Crandell feline kidney cells (CrFK). Cultures were washed twice with culture medium Cultures were washed twice with Dulbecco's modified Eagle's medium supplemented with antibiotics and 10 % fetal calf serum and incubated at 37 °C humid atmosphere with 5 % CO2. Cultures were checked daily for cytopathologic changes for a maximum of 10 days. At least three passages were made before cultures were considered negative. At the end of each passage, all cultures were tested for the presence of herpesviral DNA by PCR.

Electron microscopy

The fourth passage of PPki cells inoculated with a genital mucosa sample from dolphin 34 was fixed in 4 % formaldehyde and 1 % glutar(- di)aldehyde and postfixed in 1 % osmium tetroxide. After embedding in epoxy resin, thin sections were prepared, stained with 6 % saturated uranyl acetate and lead citrate, and examined with a Philips Morgagni 268D electron microscope (F.E.I., Brno, Czech Re- public). Formalin-fixed, paraffin-embedded biopsies of the penile lesion of dolphin 19 were deparaffinized and processed in the same manner as the PPki samples.

Histology

Biopsy samples of the penile lesion of dolphin 19 were fixed in 10 % neutral-buffered formalin, routinely processed, and embedded in paraffin. Five-micrometer-thick sections were mounted on glass slides and stained with hematoxylin and eosin (H&E).

Herpesvirus PCR and nucleotide sequencing

From the genital mucosa samples collected in lysis buffer and from the biopsies obtained from the genital lesion of dolphin 19, DNA was isolated using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, GmbH), following the protocol provided by the manufacturer. A nested herpesvirus PCR was performed as described previously [215]. In brief, two forward primers (HV-F1:5'-GAYTTYGCNAGYYTNTAYCC-3' and HV-F2: 5'-TCCTGGACAAGCAGARNYSG-CNMTNAA-3') and one reverse primer (HV- R1: 5'-GTCTTGCTCACCAGNTCNACNC-CYTT-3') were used in the first PCR. An aliquot of 2 ml from the first PCR reaction was used for a nested PCR with one forward primer (HV-F3: 5'-TGTAACTCGGTG- TAYGGNTTYACNGGNGT-3') and one reverse primer (HV-R2: 5'-CACAGAGTCC- GTRTCNCCRTANAT-3'). Products of the PCR reactions were checked by electrophoresis on a 2 % agarose gel for fragments of the correct size. Automated sequencing of PCR fragments was performed on an ABI 3130XL genetic analyzer with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) using the primers F3 and F2 for the fragments of the nested PCR and primers F1, R1, and R2 if a PCR fragment from the first PCR was available. For identification of sequenced fragments, the BLAST option of the National Center for Biotechnology Information website was used (http://www.ncbi.nlm.nih.gov/). For phylogenetic analysis, the SEQBOOT and DNAML programs of the Phylip package were used [235].

Papillomavirus PCR

Because papillomavirus is known to cause genital lesions in bottlenose dolphins [236], a papillomavirus PCR was performed on all genital mucosa samples collected in lysis buffer using primers MY11/ MY09 [237] and GP5+/GP6+ [238]. This PCR had been successful in detecting DNA of two *Phocoena spinnipensis* papillomaviruses, one of which is closely related to bottlenose dolphin papillomavirus 2 [219].

Herpesvirus serology

A Nonidet-P40 (NP-40) cell lysate was prepared using the fourth passage of PPki cells infected with the herpesvirus isolated from dolphin 34 (named TTHV). This cell lysate, diluted in phosphate-buffered saline (PBS), was used as antigen coating for enzyme-linked immunosorbent assay (ELISA). Antigen coating was incubated overnight at room temperature in 96-well ELISA plates. Checkerboard titrations with a serum pool from PCR-positive dolphins were performed to determine optimal dilution of the antigen coating and the conjugate. Serum samples were tested in serial 2 log dilutions on a coating with TTHV/NL/08-01-infected PPki cells and on a coating of uninfected PPki cells.

As a conjugate, horseradish peroxidase-labeled protein A was used. The ELISA buffer used for dilution of the sera and conjugate was PBS, 0.5 % bovine serum albumin, 1 % powdered milk, and 0.05 % Tween 20. Serum and conjugate were incubated for 1 hr at 37 °C. Tetramethylbenzidine was used as a substrate. Color reaction was stopped after 10 min using sulfuric acid, and absorbance was measured at 450 nm. Samples with a signal of 2 times the background or higher, at titers equal to or greater than 20, were considered positive. Of each of the 36 dolphins, the most recent and the oldest serum samples available were checked to investigate how long the infection had been present. Annual samples, as available, for six dolphins (dolphins 2, 21, 7, 20, 24, and 25) that had seroconverted in this time period, were checked to determine the age of seroconversion more precisely.

Retrospective analysis of necropsy reports

Gammaherpesvirus infection has been associated with the development of urogenital tumors in California sea lions [233]. Therefore, available necropsy reports of animals that died since 1993 (10 female adults, two male adults, five female juveniles or neonates, and seven male juveniles or neonates) were reviewed for diagnosis of urogenital neoplasia.

Statistical analysis

We used Fisher's exact test [239] to examine the hypotheses that 1) a positive herpesvirus PCR was independent of presence of macroscopically visible lesions in the genital mucosa and 2) the occurrence of an antibody titer was independent of age class.

RESULTS

Inspection of genital mucosa

Macroscopic lesions were observed in the penile mucosa of three of 13 male dolphins and in the vulval mucosa of four of 15 female dolphins (Table 1). These lesions were single or multiple plaques that were pale yellow to white, with an irregular shape, a smooth surface, and raised 1–7 mm (males) or 1–4 mm (females) above the surrounding mucosa (Fig. 1). The plaques ranged in diameter from 5 mm to 50 mm in males and 5 mm to 20 mm in females. In 27 of 28 dolphins, the presence of a macroscopically visible genital lesion was associated with a positive herpes PCR ($P<0.001$, Fisher's exact test; see below).

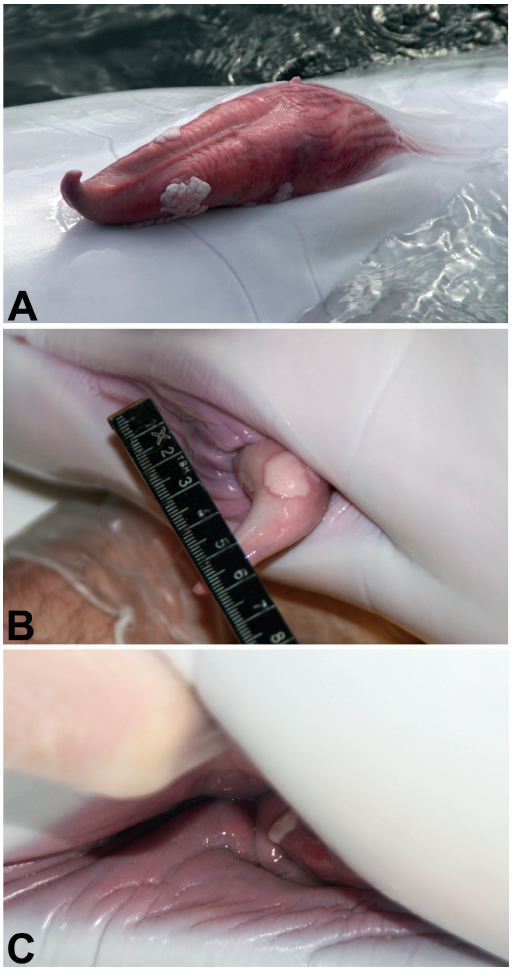

Figure 1 Plaques associated with herpesvirus infection in the genital mucosa of bottlenose dolphins. A. Adult male (34) with two plaques on penile mucosa. B. Juvenile male (19) in which plaque on penile mucosa occurred during study period. C. Juvenile female (36) with plaque on vulvar mucosa.

In most of the dolphins, genital plaques were already seen at the first observation during the study period. However, in dolphin 19, a penile plaque occurred between observations on 8 and 25 September 2008 (Fig. 1).

Virus culture

On day 3 after inoculation during the first passage, patches of cytopathic effect (CPE) were visible in PPki cell cultures of samples from dolphins 34 and 5. Over the next 4 days, this CPE became confluent. Supernatant and cells were checked by herpesvirus PCR and passaged onto fresh PPki cell cultures, resulting in CPE similar to that in the first passage. These cultures were positive for bottlenose dolphin gammaherpesvirus by PCR and sequencing of the PCR products (see below).

No herpesvirus was detected by CPE or herpesvirus PCR in PPki cells inoculated with the three samples, from dolphins 16, 26, and 35. No herpesvirus was detected by CPE or PCR in any of the five samples by use of TTki, MDBK, MDCK, or CrFK cells.

Electron microscopy

Nuclei of PPki cells inoculated with a genital mucosa sample from dolphin 34 contained many round or hexagonal particles with a consistent diameter of approximately 100 nm (Fig. 2). Some particles had a round, electron-dense core, whereas others were empty. Size, shape, and location of these particles are consistent with herpesvirus nucleocapsids [240]. Epithelial cells at the surface of the mucosal biopsy sample of the penile lesion of dolphin 19, corresponding with the hypereosinophilic layer seen by light microscopy, contained round or hexagonal unenveloped particles with a consistent diameter of approximately 100 nm and an electron-dense or electron-lucent core. In the intercellular space between adjacent epithelial cells were enveloped virus particles. Size, shape, and location of these particles are consistent with herpesvirus nucleocapsids [240]. The affected cells were characterized by fragmentation of the nucleus, condensation of chromatin, and vacuolization of the cytoplasm (Fig. 3).

Histology

The biopsy sample of the penile lesion contained the full thickness of the epithelium and was approximately 100 cells thick, with scant subjacent connective tissue. The epithelium showed moderate hyperplasia, disorganization of the histologic architecture, and prominent papillae of the lamina propria. In general, the epithelial cells were large and polygonal, with distinct borders, abundant pale eosinophilic, finely vacuolated cytoplasm and large, centrally located, and oval nuclei with one or two prominent basophilic nucleoli. Adjacent to the basement membrane, the epithelial cells had more basophilic nuclei and a few had mitotic figures, consistent with epithelial hyperplasia. In the middle layer of the epithelium, some cells had perinuclear cytoplasmic vacuolation and nuclear condensation. At the surface of the epithelium was a 10-cell-thick layer of

cells with progressive flattening, eosinophilia of the cytoplasm, and fragmentation and condensation of the nucleus. At the transition of this hypereosinophilic cell layer and the underlying epithelium, the cell nuclei had marginated chromatin and an eosinophilic core. These cores were suggestive of intranuclear inclusion bodies, but most lacked a clear halo (Figs. 4, 5).

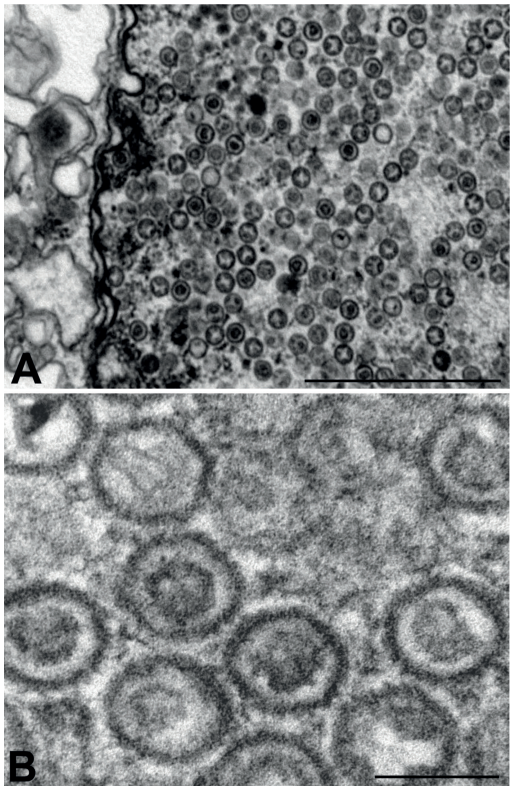

Figure 2. Transmission electron micrographs of primary cell culture of harbor porpoise kidney cells inoculated with penile plaque scraping from bottlenose dolphin 34. Uranyl acid and lead citrate stain. A. Many herpesvirus nucleocapsids are present in the nucleus. Bar=1,000 nm. B. Detail of A, showing round to hexagonal shape of nucleocapsids, some with electron-dense core. Bar=100 nm.

Herpesvirus PCR and DNA sequencing

In nine of 36 dolphins, herpesvirus was detected in genital mucosa samples by PCR and subsequent sequencing of the PCR product (Table 1). Full analysis of the PCR products of four samples (dolphins 5, 16, 19, and 34) demonstrated a 356-nucleotide fragment of the polymerase gene, showing 100 % identity with a bottlenose dolphin gammaherpesvirus from bottlenose dolphins stranded on the Florida Keys and 91 % identity with a gammaherpesvirus from bottlenose dolphins stranded on the Atlantic side of Florida or North Carolina (Fig. 6).

All organ samples of the four cases of perinatal mortality and two abortions were negative for herpesvirus by PCR.

Papillomavirus PCR

All 36 genital mucosa samples were negative for papillomavirus by PCR.

Herpesvirus serology

By ELISA using the virus isolate TTHV/ NL/08-01 as antigen, seven of 15 (47 %) juvenile dolphins had specific antibodies to bottlenose dolphin gammaherpesvirus in the most recently collected sera, all with titers >160. In comparison, 20 of 21 (95 %) adult dolphins had specific antibodies, also all with titers >160 (Table 1). The proportion of seropositive animals was significantly higher in adults than in juveniles ($P<0.001$, Fisher's exact test).

In the time between the dates of collection of the oldest and most recent sera, seroconversion occurred in seven dolphins, six of which were juveniles. Based on analysis of annual samples the youngest female to have seroconverted was 5 yr old. The youngest male was 1 yr old. The oldest female without detectable antibodies was 13 yr old, and the oldest male was 9 yr old.

Retrospective analysis of necropsy reports for diagnosis of urogenital neoplasia

Urogenital tumors were not diagnosed in any of these necropsy reports. The only tumor diagnosed was an adrenal adenocarcinoma in a geriatric male.

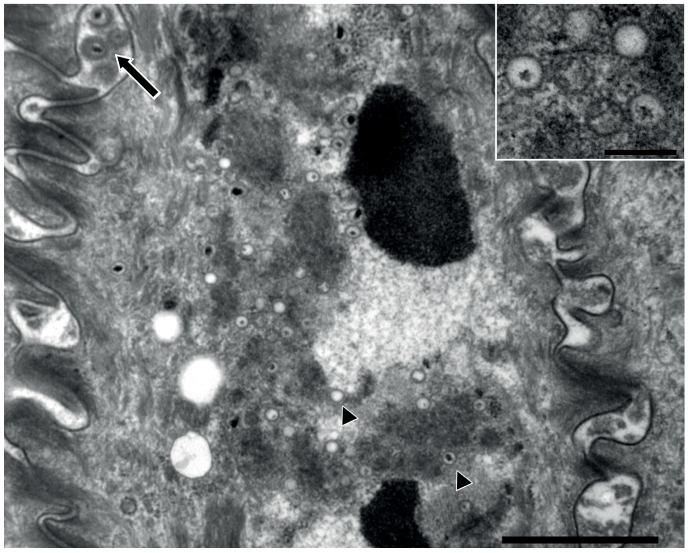

Figure 3. Transmission electron micrographs of superficial epithelial cells in biopsy of penis plaque from dolphin 19, showing unenveloped viral nucleocapsids (arrowheads) in the cell and enveloped nucleocapsids (arrow) in the intercellular space. Note the lack of nuclear membrane, the electron-dense clumps of condensed chromatin and the cytoplasmic vacuoles. Uranyl acetate and lead citrate. Bar=1,000 nm. Inset. Detail of unenveloped viral nucleocapsids with a diameter of approximately 100 nm. Bar=200 nm.

DISCUSSION

We report the first successful cultivation of a herpesvirus from bottlenose dolphins or any other cetacean species. The availability of a culture of this bottlenose dolphin herpesvirus (TTHV) opens new avenues in virus characterization, development of diagnostic methods, understanding of pathogenesis, and vaccine development for TTHV infection in bottlenose dolphins. As a first step in improving diagnosis, we developed an ELISA to detect specific antibodies in serum.

The production of infectious progeny virus by herpesviruses is accompanied by destruction of the infected cell [55]. Epithelial herpesvirus infections are therefore commonly accompanied by destruction of the epithelium, necrosis, and ulceration. Hyperplasia with prominent papillae of the lamina propria and perinuclear vacuolation is more reminiscent of lesions caused by papillomavirus [241]. However herpesvirus has been associated with hyperplasia and papillomas in Neotropical parrots [242], cloacal papillomas in parrots [243], a papilloma-like penile lesion in a Blainville's beaked whale (*Mesoplodon densirostris*; [222]), and fibropapillomatosis in marine turtles [244, 245]. Although in parrots papillomavirus was initially suspected to be causative for the papillomas, it could not be detected in two investigations into the etiology [242, 243].

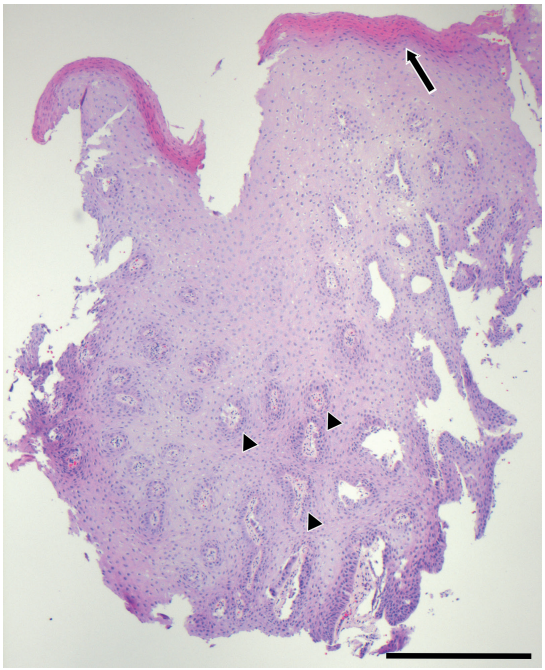

Figure 4. Biopsy of penis plaque from dolphin 19, showing epithelial hyperplasia. Note the disorganized histologic architecture, prominent papillae of the lamina propria (arrowheads) and the hypereosinophilic superficial cell layer (arrow). H&E.

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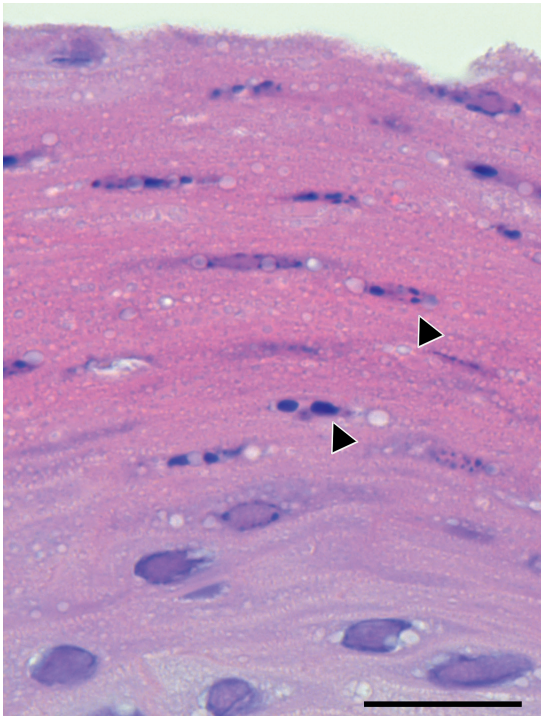

Figure 5. Detail of Figure 4, showing hypereosinophilic superficial cell layer. The cells with fragmented nuclei and condensed chromatin (arrowheads) correspond with the epithelial cells in which herpesvirus-like particles were detected by electron microscopy. H&E. Bar=20 µm.

Our study provides strong evidence that infection with TTHV/NL/08-01 causes genital plaques in bottlenose dolphins. First, in 27 of 28 dolphins, the result of herpesvirus PCR on genital samples correctly predicted the presence of genital plaques. Second, one bottlenose dolphin (19) developed a genital plaque in the same period that the genital sample became positive by herpesvirus PCR, and the products of this herpesvirus PCR were identical to that of the newly isolated TTHV. Third, biopsies of the plaque of bottlenose dolphin 19 contained herpesvirus polymerase encoding DNA and herpesvirus particles in the lesion. No other viruses were noted despite extensive examination of the sample by electron microscopy. Finally, the results of our study correspond with a previous study where a closely related or identical herpesvirus was detected by PCR in localized genital lesions of bottlenose dolphins [57]. Although papillomavirus has been associated with genital lesions in bottlenose dolphins [236] , we found no evidence of papillomavirus infection in these genital mucosa lesions. Papillomavirus DNA was not detectable by PCR in any of the samples of the genital mucosa or in the biopsied material from the penile lesion of dolphin 19. Consensus primers were used that were successful in finding *Phocoena spinnipinis* Papillomavirus type1 that is closely related to *Tursiops truncatus* Papillomavirus type 2 [219]. Detailed electron microscopic examination of the biopsy sample of the penile plaque from dolphin 19 did not reveal papillomaviruslike particles. Based on these observations coinfection with papillomavirus is unlikely. We found no evidence that TTHV in bottlenose dolphins caused disease other than genital plaques. No herpesvirus was detected in samples from six bottlenose dolphin cases of perinatal mortality and abortions, which have been associated with genital herpesvirus infection in multiple species [231]. Of 24 necropsy reports examined, there were no records of urogenital neoplasia, which has been associated with herpesvirus infection in California sea lions [233]. However, this conclusion is based on examination of a relatively small number of cases, and further studies are recommended to determine the pathogenic potential of this herpesvirus.

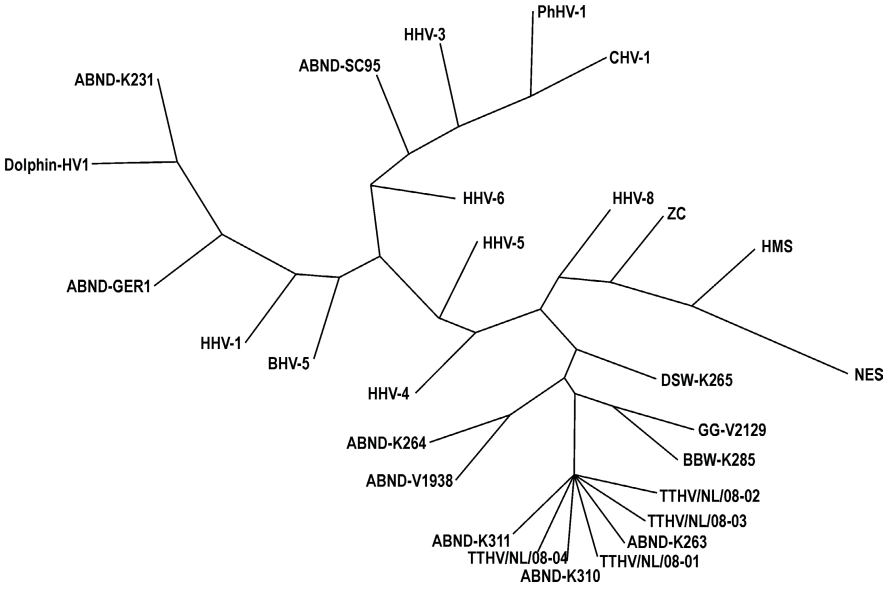

Figure 6. Unrooted phylogenetic tree of polymerase gene fragments for comparison of bottlenose dolphin herpesviruses from this study with bottlenose dolphin herpesviruses from previous studies, herpesviruses from other marine mammal species, and representative human and canine herpesviruses. Nucleotide sequences were subjected to bootstrapped (n=500) maximum likelihood analysis using the Phylip package. The consensus tree is shown. GenBank accession numbers are given in parentheses: ABND-K263, bottlenose dolphin K263, USA captive (AY952777); ABND-K310, bottlenose dolphin K310 tongue lesion, USA stranded Florida Keys (AY952779); ABND-K311, bottlenose dolphin K311, USA captive (AY949831); DSW-K265, dwarf sperm whale (Kogia simus) K265, USA (AY949830); ABND-V1938, bottlenose dolphin V1938, USA stranded North Carolina (DQ288667); ABND-K264, bottlenose dolphin K264, USA stranded Jacksonville Florida (AY952776); HHV-4, Human herpesvirus 4, EBV (AJ507799); BBW-K285, Blainville's beaked whale (Mesoplodon densirostris), USA stranded Kure Beach, North Carolina (AY949828); GG-V2129, Risso's dolphin (Grampus griseus), USA stranded Gulf of Mexico Florida, 2005 (DQ288666); NES, northern elephant seal (Mirounga angustirostris), USA (DQ183057); HMS, Hawaiian monk seal (Monachus schauinslandi), USA (DQ093191); ZC, California sea lion (Zalophus californianus), USA (AF236050); HHV-8, Human herpes virus 8 (AF148805); TTHV/NL/08-01, Bottlenose dolphin 34, Netherlands, captive (GQ258353); TTHV/NL/08-02, bottlenose dolphin 5, Netherlands, captive (GQ258354); TTHV/NL/08-03, bottlenose dolphin 16, Netherlands, captive (GQ258355); TTHV/NL/08-04, bottlenose dolphin 19, Netherlands, captive (GQ258356); CHV-1, Canine herpesvirus 1 (AY949827); PhHV-1, Phocine herpesvirus type 1, Pacific (U92269); HHV-3, Human herpesvirus 3, VZV (X04370); ABND-SC95, bottlenose dolphin, USA (AF245443); BHV-5, bovine herpesvirus 5, strain SV507/99 (AY261359); HHV-1, human herpesvirus 1, HSV-1, (AB070848); ABND-GER1, bottlenose dolphin, Germany (AY608707); DOLPHIN-HV1, bottlenose dolphin, USA, Hilton Head Island South Carolina, 1995 (AF196646); ABND-K231, bottlenose dolphin K231, USA (captive) (AY949832); HHV-5, Human herpesvirus 5, HCMV (M14709); and HHV-6, Human herpesvirus 6 (AB283024).

The degree of genetic similarity of TTHV from this study to previously detected herpesviruses from bottlenose dolphins seems to be related to the geographic origin of the dolphins. A herpesvirus from a bottlenose dolphin stranded on the Florida Keys [57], which lies adjacent to the Gulf of Mexico (origin of dolphin 5 and 16), and Cuban waters (origin of dolphin 34), showed 100 % identity to the herpesvirus from our study. In contrast, two herpesviruses from bottlenose dolphins stranded further north, on the Atlantic coasts of Florida and North Carolina [57], showed only 91 % identity with the herpesvirus from our study. Because herpesviruses may show a marked geographically defined genotype distribution [55], genetic differences between herpesviruses from bottlenose dolphins may provide information on the degree of separation between the populations from which they originate.

Results of serologic examination suggest that the herpesvirus is endemic in this zoo collection of dolphins and is transmitted by sexual contact. Transmission by sexual contact is suggested by the correlation between minimum age at which sera are positive (1 yr for males, 5 yr for females [Table 1]) and onset of sexual activity. From the age of less than 1 yr, males insert their penis into the genital slit of adult females and that of males of all ages (unpublished data). In contrast, females are only penetrated when they become sexually mature, at a minimum age of 4 yr [234]. Seroconversions occurred throughout the period 1997 to 2007, and antibodies also were demonstrated in the oldest available sera from 1994, indicating the virus is endemic in this collection.

In conclusion, we report the first successful cultivation of a herpesvirus from bottlenose dolphins. This TTHV belongs to the subfamily of *Gammaherpesvirinae* and is significantly associated with genital plaques in both males and females. Seroepidemiology using a newly developed ELISA demonstrated infection is endemic in the zoo collection and apparently spreads by sexual contact. We found no evidence that TTHV is associated with perinatal mortality or urogenital neoplasia. However, more extensive studies are warranted to confirm or negate these preliminary observations.

ACKNOWLEDGMENTS

We acknowledge the training staff of Dolfinarium Harderwijk and Parc Asterix and Vivian Emmer for assistance with obtaining and processing of the samples and the inspection of the animals.

White-beaked dolphin, Dolfinarium Harderwijk, the Netherlands

6

IS DOLPHIN MORBILLIVIRUS VIRULENT FOR WHITE-BEAKED DOLPHINS *(LAGENORHYNCHUS ALBIROSTRIS)*?

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ABSTRACT

The virulence of morbilliviruses for toothed whales (odontocetes) appears to differ according to host species. In 4 species of odontocetes, morbilliviruses are highly virulent, causing large-scale epidemics with high mortality. In 8 other species of odontocetes, including white-beaked dolphins (*Lagenorhynchus albirostris*), morbilliviruses have been found as an incidental infection. In these species, the virulence of morbilliviruses is not clear. Therefore, the admission of 2 white-beaked dolphins with morbillivirus infection into a rehabilitation center provided a unique opportunity to investigate the virulence of morbillivirus in this species. By phylogenetic analysis, the morbilliviruses in both animals were identified as a dolphin morbillivirus (DMV) most closely related to that detected in a white-beaked dolphin in Germany in 2007. Both animals were examined clinically and pathologically. Case No. 1 had a chronic neural DMV infection, characterized by polioencephalitis in the cerebrum and morbillivirus antigen expression limited to neurons and glial cells. Surprisingly, no nervous signs were observed in this animal during the 6 months before death. Case No. 2 had a subacute systemic DMV infection, characterized by interstitial pneumonia, leucopenia, lymphoid depletion, and DMV antigen expression in mononuclear cells and syncytia in the lung and in mononuclear cells in multiple lymphoid organs. Cause of death was not attributed to DMV infection in either animal. DMV was not detected in 2 contemporaneously stranded white-beaked dolphins. Stranding rate did not increase in the region. These results suggest that DMV is not highly virulent for white-beaked dolphins.

INTRODUCTION

Morbilliviruses are recently detected pathogens of marine mammals that cause epidemics with high mortalities. Virulence and susceptibility for morbillivirus as well as epidemiology differ among species. For example, phocine distemper virus causes mass mortality in harbor seals (*Phoca vitulina*), whereas gray seals (*Halichoerus grypus*) do not develop clinical signs of disease [66]. For many odontocete species, including the white-beaked dolphin (*Lagenorhynchus albirostris*), virulence and epidemiology of morbillivirus are unclear.

The virulence of morbillivirus for odontocetes appears to differ according to host species. They have been responsible for epidemics with high mortalities in bottlenose dolphins (*Tursiops truncatus*), common dolphins (*Delphinus delphis*), long-finned pilot whales (*Globicephala melas*), and striped dolphins (*Stenella coeruleoalba*) [67-70, 114]. Morbillivirus infection causes lesions in odontocetes in 2 ways. First, it is a lymphotropic virus, which is highly immunosuppressive and thereby allows preexisting infections to be exacerbated and secondary infections to spread. Second, from the lymphoid tissues, the virus may spread to other tissues, such as the brain and the epithelia of multiple organs, where it

is characterized by viral inclusions both in the cytoplasm and the nucleus. Particularly in the brain, infection is also associated with inflammation, necrosis, and demyelinization [68]. Clinical signs in cetaceans have been observed in striped dolphins and common dolphins. In striped dolphins, poor body condition, disorientation, apathy, muscle tremors, abnormal respiratory rates, ulcers in the mouth, and increased parasite burden of the skin were found. In common dolphins, seizures, uncontrolled trembling, and dyspnea have been observed [66]. In contrast, to date, morbilliviruses have been observed as incidental pathogens in 3 species: harbor porpoises (*Phocoena phocoena*) [72], white-beaked dolphins [73, 74], and a pygmy sperm whale (*Kogia breviceps*) [75]. In addition, serologic evidence for contact with morbillivirus has been found in 5 species of odontocetes: Risso's dolphin (*Grampus griseus*), Atlantic white-sided dolphins (*Lagenorhynchus acutus*), Fraser's dolphins (*Lagenodelphis hosei*), Atlantic spotted dolphins (*Stenella frontalis*), and false killer whales (*Pseudorca crassidens*) [71]. The virulence of morbillivirus for these latter 8 species, including the white-beaked dolphin, is not clear.

In odontocetes, morbillivirus infections have resulted in epidemics with high mortalities and have occurred as unique events in bottlenose dolphins and common dolphins involving a single species [47, 67, 114]. In the Mediterranean Sea, recurrent epidemics have occurred in striped dolphins with simultaneous infection of long-finned pilot whales [68-70]. In these epidemics, it is assumed an infection of an immunonaive population has occurred. The source of infection is not identified in any of the epidemics in odontocetes. Morbillivirus needs a large population to remain endemic. Infections of immunonaive populations should therefore be expected from large populations where morbillivirus is endemic. The population structure and serologic status of white-beaked dolphins is therefore important. White-beaked dolphins inhabit the North Atlantic Ocean, and based on genetic analysis, there are 2 separate populations in the eastern North Atlantic[246]. One large population resides in the North Norway-Barents Sea (up to 100 000 animals) and a smaller one occurs around the United Kingdom and in the North Sea (22 000 animals). Vagrant animals occur as far south as the Strait of Gibraltar and the Mediterranean Sea [247]. There is no knowledge of the epidemiology of morbillivirus in white-beaked dolphins.

The white-beaked dolphin is the second most common species to strand on the North Sea coasts of the Netherlands and adjacent countries. Autopsy programs exist and stranding data are collected in the Netherlands, Belgium, and Schleswig-Holstein. Schleswig-Holstein is the northernmost state of Germany, close to the Netherlands and the state where a white-beaked dolphin infected with dolphin morbillivirus (DMV) was found in 2007 [73]. From 2000 to 2012, 4.6 animals stranded on average annually on these coasts. Around 25 of these carcasses were autopsied and evaluated for presence of morbillivirus (U. Siebert, T. Jauniaux, and T. Kuiken, personal communications, 2013).

In 2011, 2 white-beaked dolphins stranded alive on the Dutch coast and died or were euthanized after spending a period of time at the rehabilitation center SOS-Dolfijn in

Harderwijk. The diagnosis of morbillivirus infection in both animals provided a unique opportunity to gain information both on clinical and pathological aspects of morbillivirus infection in this species.

The primary goal of our research was to fully investigate the virulence of morbillivirus in white-beaked dolphins. This report describes the clinical signs, gross autopsy, histology, immunohistochemistry, bacteriology, and serology of the 2 dolphins admitted into the rehabilitation center. Two more white-beaked dolphins stranded on the Belgian and Dutch coasts within a month of the last arrival in the rehabilitation center. We investigated samples of these last 2 animals for the presence of morbillivirus infection and found they were both uninfected. The secondary goal was to investigate the epidemiology of morbillivirus in white-beaked dolphins and other odontocetes by comparing the RNA sequence of the morbillivirus of white-beaked dolphins with RNA sequences of previously found odontocete morbilliviruses.

MATERIALS AND METHODS

Signalment

Case No. 1 was a juvenile male that was 229 cm long and weighed 153 kg. It stranded alive on the island Ameland, the Netherlands, on June 12, 2011, and died on December 12, 2011, while in rehabilitation. Based on length and sexual immaturity, its age was between 4 and 8 years old [100].

Case No. 2 was a juvenile female that was 158 cm long and weighed 49 kg. It stranded alive at Den Helder, the Netherlands, on December 4, 2011, and was humanely killed the next day. Based on her length, it was estimated to be 18 months old [100].

Case No. 3 was a subadult male that was 257 cm long and weighed 255 kg. It stranded dead at Zoutelande, the Netherlands, on January 3, 2012. The carcass was fresh.

Case No. 4 was an adult female that was 211 cm long and weighed 240 kg. It stranded alive at Koksijde, Belgium, on December 17, 2011. Due to the presence of severe lesions, the animal was euthanized.

Clinical Evaluation

SOS-Dolfijn is a rehabilitation center for small odontocetes. It has two 50-m^3 pools with fresh water from a local source, to which salt is added. Salinity was kept between 20 and 30 parts per thousand (ppt) until September 23, 2011, and between 25 and 35 ppt afterwards. Water treatment is by high-rate sand filtration with a turnover time of approximately 1 hour for the entire volume of each pool. Water temperature varies from 11 to 21 °C. Upon admittance, new animals are subjected to a full veterinary examination by an experienced marine mammal veterinarian. The monitoring of complete blood count and clinical

chemistry of blood samples is done daily after admittance and thereafter according to perceived necessity. In the first period of rehabilitation, animals are observed continuously day and night. Multiple observations on respiration rate, defecation, cramps, food intake, and body temperature are recorded. In addition, a log book is kept in which swimming behavior, alertness, and other observations that are presumed potentially relevant are recorded. As the animal improves, observation and care diminish to a minimum of 9 hours per day. Case No. 1 was continuously observed after admittance for 10 days. Case No. 1 was moved to a larger outdoor pool (120 m^3) on September 14, 2011, in preparation for its release. Case No. 2 was continuously observed from admittance until euthanasia.

Autopsy, Histology, and Immunohistochemistry

Autopsies were done on case Nos. 1 and 2 according to a standard protocol [89]. The following tissues were sampled for histology: adrenal gland, bronchus, cerebellum, cerebrum, colon, duodenum, esophagus, forestomach, fundic stomach, gonads, heart, jejunum, kidney, liver, lung, mesenteric lymph node, muscle, pancreas, pulmonary lymph node, pyloric stomach, skin, spleen, thymus, thyroid, trachea, tracheobronchial lymph node, and urinary bladder. Tissue samples were fixed in 10 % neutral-buffered formalin, routinely processed, and embedded in paraffin. The 3µ-m-thick sections were mounted on glass slides and stained with hematoxylin and eosin (HE) for light microscopy.

For detection of morbillivirus, 3-µm-thick sections of all tissues sampled for histology were stained with an immunohistochemical technique as follows. After antigen retrieval (boiling for 15 minutes in citric acid 10 mM, pH 6.0), slides were incubated with 3 % hydrogen peroxide in phosphate-buffered saline solution (PBS) for 10 minutes to inactivate endogenous peroxidase. To block nonspecific antibodies, the sections were incubated with 0.1 % bovine serum albumin (BSA) in PBS for 10 minutes. Then the slides were incubated with a monoclonal antibody directed against the nucleoprotein of canine distemper virus (MoAb CDV-NP isotype IgG2b; VMRD, Pullman, WA) 1:400 in PBS/0.1 % BSA for 1 hour at room temperature. This antibody is known to cross-react with DMV antigen and has been used previously in DMV-infected tissues of cetaceans [248]. After being washed with PBS-Tween, the sections were incubated for 30 minutes at room temperature with a biotinylated secondary antibody (a-mouse-bio; DAKO, Glostrup, Denmark), 1:100 in PBS/0.1 % BSA. After washing, the slides were incubated with ABComplex (DAKO) for 1 hour at room temperature. Antibody binding was visualized using 3-amino- 9-ethylcarbazole (AEC) and hydrogen peroxide. The sections were counterstained with hematoxylin. In the staining procedure, an isotype IgG2b control was included as a negative control and a sample of the prostate of a canine distemper virus–infected Caspian seal (Pusa caspica) was included as a positive control.

Bacteriology

For bacteriological examination, samples of lung, kidney, liver, spleen, pulmonary lymph node, and adrenal gland were frozen at –20 °C and transferred on dry ice to Inverness, Scotland, where they were cultured according to a standard protocol. Briefly, each tissue was plated on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK), MacConkey agar (Oxoid), and Farrells medium [153], which was set up specifically for the recovery of Brucella ceti [154]. CSBA and Farrells plates were incubated aerobically plus 5 % CO2 and examined daily for 14 days, whereas MacConkey agar plates were incubated aerobically without added CO2 at 37°C for 48 hours.

RT-PCR, Sequencing, and Phylogenetic Analysis

Tissue samples were fixed in RNAlater solution (Life Technologies Corporation, Carlsbad, CA) until analysis. The following tissues were tested: for case No. 1, adrenal gland, prescapular lymph node, trigeminal nerve, spleen, lung, kidney, urinary bladder, liver, and brain; for case No. 2, urinary bladder, kidney, trigeminal nerve, liver, spleen, lung, brain, adrenal gland, tongue, pulmonary lymph node, stomach ulcer, blowhole swab, tongue ulcer swab, and genital slit swab; for case No. 3, lung, urinary bladder, cerebellum, and kidney; and for case No. 4, lung, urinary bladder, kidney, spleen, pulmonary lymph node, and brain.

Total RNA was isolated from 300 ml of a 10 % organ homogenate using the High Pure Viral Nucleic Acid Kit (Roche diagnostic GmbH, Mannheim, Germany), following the protocol provided by the manufacturer. For reverse transcriptase polymerase chain reaction (RT-PCR), morbillivirus- specific primers P1: 50-ATGTTTATGATCACAGCGGT-30 and P2: 50-ATTGGGTTGCACCACTTGTC-30 were used after first-strand synthesis with specific morbilliviral primers. PCR reactions were checked on agarose gels. Automated sequencing of RT-PCR fragments was performed on an ABI 3130XL genetic analyzer with the Big Dye terminator cycle sequencing kit (ABI, Applied Biosystems, Foster City, CA) using the RT-PCR primers P1 and P2. For identification of sequenced fragments, the BLAST option of the NCBI website was used (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis and construction of phylogenetic trees was conducted using MEGA version 5.31

Serology

Serum samples were frozen at –20 °C until analysis and tested for the presence of morbillivirus antibodies by a virus neutralization assay as previously described [249]. In brief, 2-fold dilutions of heat-inactivated serum samples were incubated with 100 median tissue culture infectious dose of CDV. After a 1-hour incubation at 37 °C, 104 Vero cells were added to each well. After 4 to 6 days, the plates were checked for the presence of cytopathic effect (CPE). Antibody titers were expressed as the reciprocal of the highest serum dilution with complete inhibition of CPE. Antibody titers >10 were considered positive [250, 251].

RESULTS

History and Clinical Signs

Upon arrival on June 12, 2011, case No. 1 was in poor nutritional condition and needed continuous support. Frequent tremors and occasional cramps were noted during the first month. After the first week, the dolphin managed to stay afloat without support, and its swimming behavior gradually improved.

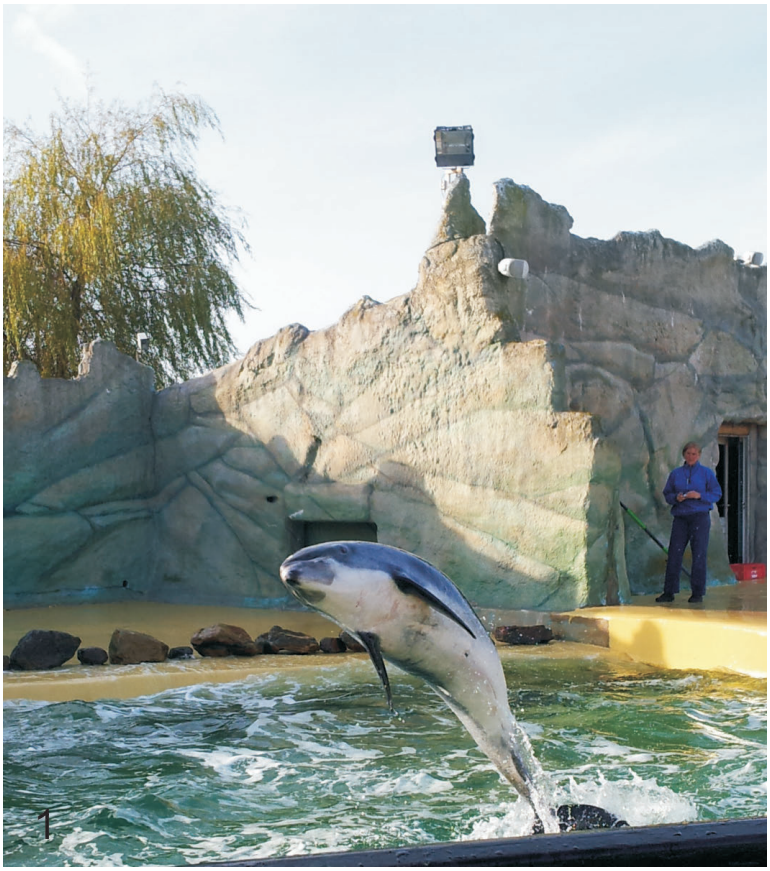


Figure 1. White-beaked dolphin, case No. 1.

Normal swimming was observed 5 weeks after admission. Initial complete blood count and clinical chemistry indicated the dolphin had a mild anemia, muscular damage, and an inflammation based on increased number of white blood cells (WBCs), fibrinogen level, and sedimentation rate. Gastroscopy revealed that foreign materials, mainly pieces of plastic, were present in the forestomach. The animal received various antimicrobial drugs until July 1 (18 days postadmission), when it was considered healed from the previously noted inflammation. By July 22 (40 days postadmission, 21 days after initial treatment had stopped), WBC numbers had increased again. A regular tremor of the dorsal fin and signs of pneumonia, notably inactivity and forced labored breathing with sporadic exudates upon expiration, were noted. Antibiotic therapy was restarted and clinical signs subsided after 17 days. WBC levels normalized toward the end of August. Subsequent to the suspected pneumonia, a dermatitis developed in September, and antibiotic treatment continued until the skin appeared healed by December 7. The dermatitis was characterized by epidermal damage, which was irregular, multifocal to coalescing across the entire body with foci on the peduncle and head, around the dorsal fin, and between the 2 pectoral fins at the time of its severest manifestation. The dermatitis was not associated with the introduction of a new medication, nor did it improve after withdrawal of a medication. It disappeared during long-term antibiotic treatment. The animal had active behavior (Fig. 1), interest in its environment, spontaneous frequent vocalizations, and ravenous appetite with the exception of the first 2 weeks after arrival and during the start of the episode of presumed pneumonia in mid-July. On December 7, it was considered completely healthy and the final preparations for release were started, including withdrawal of antibiotics. However, on December 11, it had a dramatic drop in appetite, and antibiotic treatment was resumed. The next day, the pool was drained for diagnostic examination and treatment of the dolphin, which received intramuscular injections with midazolam (10 mg) and cefovecin (1280 mg). During these injections, the animal arched its back, stopped breathing, and died.

Case No. 2 was in very poor nutritional condition on arrival, December 4, 2011. Its breathing frequency was close to normal during transport (20/5 minutes) but became high in the rehabilitation center (43/5 minutes). Complete blood count and clinical chemistry upon admittance showed a severe leucopenia, slightly increased sedimentation rate and fibrinogen level, a mild anemia, and muscular damage. It was provided parttime support to stay afloat. Force feeding was problematic, and upon the first attempt, fish gruel was noted to be expulsed from the blowhole. The animal had interest in its surroundings and the persons accompanying it and made many vocalizations. Because of the very poor nutritional condition, leucopenia, and poor future prospects, the animal was humanely killed on December 5.

Gross Autopsy

Case No. 1 was 229 cm long (straight line from tip of snout to notch between tail flukes) and 153 kg at autopsy. Case No. 1 was in good nutritional condition based on the lack of indentation in the neck and 20-mm-thick blubber layer lateral and just cranial to the dorsal fin. There was pulmonary edema, characterized by heavy, slightly firm lungs; thick yellow-white foam in the bronchi and trachea; and white viscous fluid in the trachea and bronchi. There was edema in the mediastinum dorsal to the heart over an area of 4 by 4 cm. The gastric (64 g, 0.04 % wt/body wt), left prescapular (72 g, 0.05 % wt/body wt), right prescapular (82 g, 0.05 % wt/body wt), and pulmonary lymph nodes (78 g, 0.05 % wt/ body wt) appeared enlarged. In the forestomach, plastic debris and nylon strings were found. The compressed volume of this debris was approximately half a liter. The skin had an irregular surface due to fresh and old skin peelings mainly on the belly, peduncle, and dorsal fin.

Case No. 2 was 158 cm long (straight line from tip of snout to notch between tail flukes) and 49 kg at autopsy. Case No. 2 was in poor nutritional condition based on the indentation in the neck behind the skull. The blubber layer lateral and just cranial to the dorsal fin was 11 mm thick. Fibrinosuppurative bronchopneumonia was present, characterized by yellow viscous floccules (<2 mm diameter) in the trachea and bronchi. Pus extruded from airways upon pressure on the ventral parts of the caudal lung lobes. There was subdermal emphysema over 30 % of the body surface. The gas bubbles were small (1–5 mm diameter) and coalesced sporadically to bubbles of 2 cm diameter. There were gas bubbles in the epidermis, which caused a split in the epidermis of 10 by 5 cm. An ulcer (4 mm diameter) was found on the ventral surface of the tongue, and on the junction between the fore- to second stomach, 4 ulcers (2–3 mm diameter) were observed in the forestomach. The left prescapular (23 g, 0.05 % wt/body wt), right prescapular (23 g, 0.05 % wt/ body wt), and pulmonary (69 g, 0.15 % wt/body wt), pericardial, esophageal, and subcutaneous lymph nodes appeared to be enlarged.

Histology and Immunohistochemistry

Case No. 1 had multifocal moderate polioencephalitis in the cerebrum, characterized by perivascular aggregation of lymphocytes (Fig. 2A), neuronal necrosis, neuronophagia, vacuolation of the neuropil, and diffuse gliosis (Fig. 3A). In addition to the above chronic lesion, there were acute lesions in other organs: moderate diffuse pulmonary edema, moderate multifocal gastric hemorrhage, and mild focal thymic hemorrhage.

Morbillivirus antigen expression was limited to particulate matter in areas of polioencephalitis (Fig. 2B), as well as rare neurons and glial cells in the gray matter of the cerebrum (Fig. 3B). Most of these neurons appeared normal; others were degenerate and surrounded by phagocytes (neuronophagia).

Case No. 2 had multifocal mild interstitial pneumonia, characterized by multiple small

aggregates of large mononuclear cells—some of which formed syncytia containing 2 or 3 nuclei—in the alveolar and bronchiolar lumina of the lung (Fig. 4A). There was marked lymphoid depletion in the spleen, pulmonary lymph node, and mesenteric lymph node. In contrast, the prescapular lymph node showed lymphoid hyperplasia, characterized by homogeneous fields of lymphocytes and lymphoblasts in the cortex.

Superimposed on the above small aggregates of mononuclear cells was a marked acute aspiration pneumonia, characterized by coalescing large foci of inflammation centered on bronchi and bronchioles and consisting of many neutrophils and macrophages, mixed with cellular debris, fibrin, and few erythrocytes. There were large segments of autolytic skeletal muscle (aspirated fish remains) and aggregates of bacilli and cocci in the lumina of affected bronchi and large bronchioles. No other significant lesions were detected in any of the tissues examined from case No. 1 or 2.

Morbillivirus antigen expression occurred in a moderate number of mononuclear cells and syncytia in alveolar and bronchiolar lumina of the lung (Fig. 4B), rare mononuclear cells in the red or white pulp of the spleen, rare mononuclear cells in the cortex of the pulmonary lymph node, rare mononuclear cells in the cortex of the mesenteric lymph node, and rare enterocytes in the mucosa and mononuclear cells in the lamina propria of the intestine. No morbillivirus antigen expression was detected in any of the other tissues examined from case No. 1 or 2.

RT-PCR, Sequencing, and Phylogenetic Analysis

The brain sample of case No. 1 contained morbilliviral RNA; the remaining samples (adrenal gland, prescapular lymph node, trigeminal nerve, spleen, lung, kidney, urinary bladder, and liver) were negative. The lung of case No. 2 contained morbilliviral RNA; the remaining samples (urinary bladder, kidney, spleen, pulmonary lymph node, and brain) were negative. All samples of case No. 3 (lung, urinary bladder, cerebellum, and kidney) and case No. 4 (lung, urinary bladder, kidney, spleen, pulmonary lymph node, and brain) were negative.

The morbilliviruses observed in case Nos. 1 and 2 were identified as DMV and were most closely related to one another and to a previously observed DMV in a white-beaked dolphin in 2007. The next closest identity of the DMV found in white-beaked dolphins was with the DMV observed in striped dolphins during the 1990 epidemic in the Mediterranean Sea. Of the 299 base pairs sequenced, the DMVs found in case Nos. 1 and 2 differed in 2 and 3 locations, respectively, from the DMV of a white-beaked dolphin in 2007 and in 5 locations from the DMV of striped dolphins in 1990 (Fig. 5).

Bacteriology

In case No. 1, few *Escherichia coli* were recovered in the lung and the pulmonary lymph node, plus a mixed culture (few bacteria) in the prescapular lymph node. In case No. 2, a moderate number of *E. coli* and *Enterococcus sp.* were recovered in the lung, a single *E. coli* in the liver, a few *E. coli* and a mixed culture (few bacteria) in the spleen, and a few coagulase-negative *Staphylococcus sp.* in the pulmonary lymph node. Further identification of the mixed cultures with few bacteria was not pursued, because the colonies present were typical of organisms that most likely represented postmortem contamination.

Serology

Case No. 1 had an antibody titer against morbillivirus of 15 in samples obtained on June 13 and 14 and December 12, 2011. Case No. 2 had a titer of 40 on December 4 and 30 on December 5, 2011.

Primary Diagnoses

The primary diagnosis in case No. 1 was anaphylactic shock, based on temporal correlation of its sudden death with intramuscular midazolam and cefovecin injection, pulmonary edema, mediastinal edema, and acute hemorrhages in the stomach and thymus. The primary diagnosis in case No. 2 was aspiration pneumonia, based on observed problems with force feeding 1 day before euthanasia, fibrinosuppurative pneumonia associated with fish remains in airways, and mixed culture of *E. coli* and *Enterococcus sp*. from the lung.

DISCUSSION

In this study, we diagnosed DMV infection in 2 white-beaked dolphins, 1 subacute case and 1 chronic case. The different stages of pathogenesis were reflected by the differences in tissue tropism and associated lesions. We also ascertained that the DMV identified from these white-beaked dolphins was distinct from those from other odontocete species.

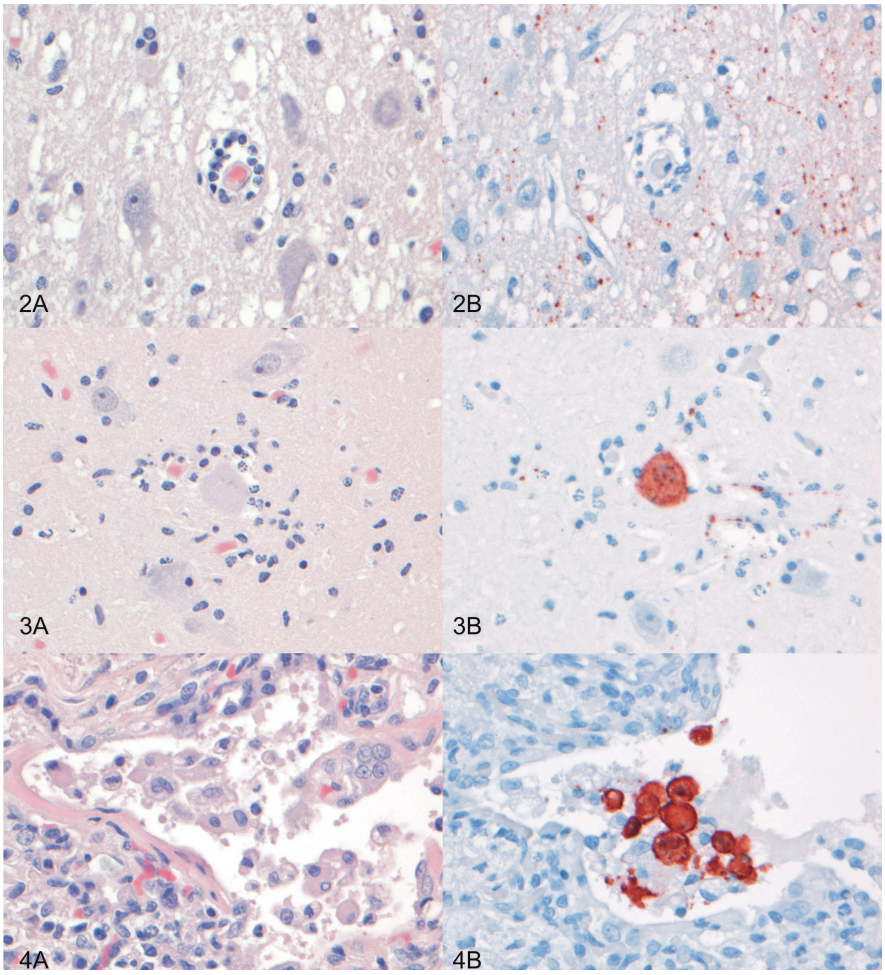
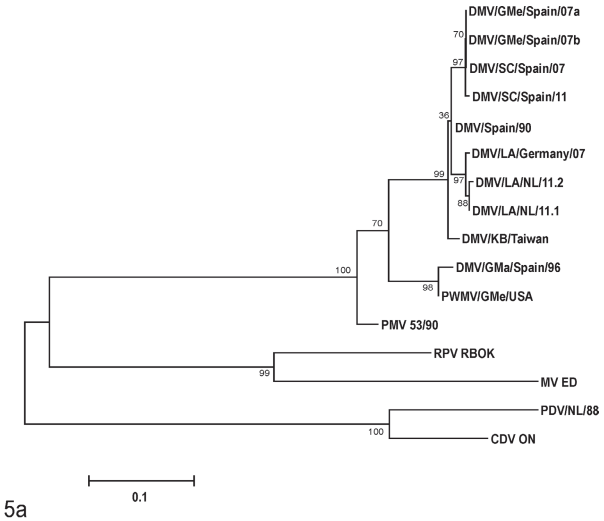
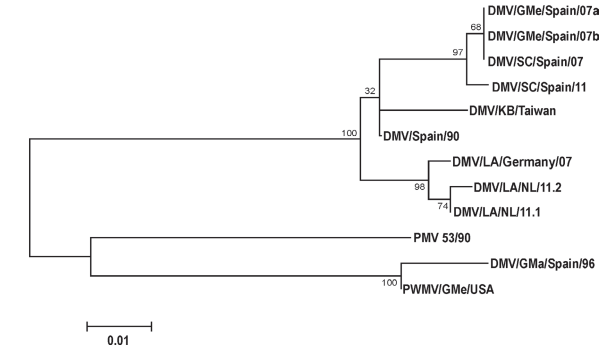


Figure 2. Cerebrum, left frontal lobe; white-beaked dolphin, case No. 1. (A) Perivascular cuffing around blood vessel in gray matter, in which there is loss of neurons, neuropil vacuolation, and diffuse gliosis. Hematoxylin and eosin (HE) stain. (B) Serial section with dolphin morbilliviral antigen in particulate form. Immunohistochemistry and hematoxylin. **Figure 3.** Cerebrum, left frontal lobe; white-beaked dolphin, case No. 1. (A) Neuronophagia characterized by degenerate neuron surrounded by phagocytes. HE stain. (B) Serial section with dolphin morbilliviral antigen in neuron. Immunohistochemistry and hematoxylin**. Figure 4**. Lung; white-beaked dolphin, case No. 2. (A) Presence of mononuclear cells and a syncytium in the lumen of a bronchiole. HE stain. (B) Serial section with dolphin morbilliviral antigen in many of the mononuclear cells. Immunohistochemistry and hematoxylin.



5a



5b

Figure 5. Maximum likelihood phylogram of a phosphoprotein (P) gene fragment of morbilliviruses (A) and cetacean morbilliviruses (B). Boot- strapping was performed with 1000 replicates using MEGA 5.31 Genbank accession numbers are given in parentheses: MV ED, measles virus Edmonston strain (K01711); RPV RBOK, Rinderpest virus RBOK strain (X68311); CDV ON, canine distemper virus Onderstepoort strain (AF305419); PDV/NL/88, phocine distemper virus, the Netherlands, 1988 (AF525289); PMV 53/90, porpoise morbillivirus, isolate 53/90 (KF650727); DMV/GMe/Spain/07a, dolphin morbillivirus, long-finned pilot whale (Globicephala melas), Spain, 2007 (HQ829972); DMV/GMe/ Spain/07b, dolphin morbillivirus, long-finned pilot whale, Spain, 2007 (EU039963); DMV/SC/Spain/07: dolphin morbillivirus, striped dolphin (Stenella coeruleoalba), Spain, 2007 (HQ829973); DMV/SC/Spain/11, dolphin morbillivirus, striped dolphin, Spain, 2011 (JN210891); DMV/SC/ Spain/90, dolphin morbillivirus, striped dolphin, Spain, 1990 (Z47758); DMV/LA/Germany/07, dolphin morbillivirus, white-beaked dolphin (Lagenorhynchus albirostris), Germany, 2007 (EF451565); DMV/KB/Tai- wan, dolphin morbillivirus, pygmy sperm whale (Kogia breviceps), Taiwan (AF333347); DMV/GMa/Spain/96, dolphin morbillivirus, short-finned pilot whale (Globicephala macrorhynchus), Canary Islands, 1996 (FJ842381); PWMV/GMe/USA, dolphin morbillivirus, long-finned pilot whale, New Jersey, USA (AF200817); DMV/LA/NL/11.1, dolphin morbillivirus, white-beaked dolphin case No. 1, the Netherlands, 2011 (KC888945); DMV/LA/NL/11.2, dolphin morbillivirus, white-beaked dolphin case No. 2, the Netherlands, 2011 (KC888946).

In case No. 1, DMV infection was diagnosed in the cerebrum and not in the cerebellum or brainstem, which corresponds with chronic cases of DMV infection in the central nervous system (CNS) of striped dolphins 4 years after the 1990 DMV epidemic in the Mediterranean Sea [76]. Surprisingly, despite the extensive DMV-associated polioencephalitis in case No. 1, behavior appeared completely normal (Fig. 1). Possibly the tremor of the dorsal fin noted at the start of its rehabilitation and during his period of illness from July 22 until August 8 was due to cerebral infection. If this animal had been autopsied without knowledge of its clinical history, the effects of the observed encephalitis would have been judged to be far graver. Throughout its entire stay in the rehabilitation center, continuous medication with antibiotics was necessary. Each attempt to stop resulted in overt clinical signs. It might well be that long-term immunosuppression by DMV infection was responsible for this dependence on antibiotics. Immunosuppression due to CDV infection in dogs may last well after virus clearance [252]. In macaques, measles virus targets memory T cells and follicular B cells, which causes temporary immunologic amnesia and explains the long-term immunosuppression [253]. In case No. 1, the antibody titer against morbillivirus remained relatively low, although a chronic CNS infection was present. This is contrary to what is found in other species, for example, in rats and humans [254, 255]. DMV antigen was observed in glial cells, which means the virus did not escape contact with the host's immune system. A possible explanation is compromised immune response from morbillivirus infection, as has been suggested for phocine distemper virus infection in harbor seals, where out of 22 harbor seals diagnosed with phocine distemper virus infection by PCR and/or immunohistochemistry on tissues, only 8 had the IgG antibody to morbillivirus. This includes 6 seals that were positive in the brain, of which only 1 had IgG antibody to morbillivirus [64]. Case No. 1 was 6 months in the facility before it died and had close contact after initial quarantine with harbor porpoises. This raises the question if DMV infection might have occurred after admittance. Several observations make this an unlikely event. Neither DMV nor porpoise morbillivirus (PMV) have been observed in the 45-year history of the rehabilitation center. Porpoise morbillivirus is highly different from the virus strains found in white-beaked dolphins (Fig. 5). The only morbillivirus known to occur in harbor porpoises is PMV, which is distinct from the DMV isolates found in white-beaked dolphins (Fig. 5). Five harbor porpoises that died in the period when case No. 1 was present at the facility tested negative for morbillivirus by PCR on samples of lung, kidney, brain, and urinary bladder, while the remaining harbor porpoises tested negative for serum antibody to morbillivirus by virus neutralization (unpublished data). Based on the chronicity of its lesions, case No. 2 was in the facility too short to have acquired DMV infection there. In case No. 2, DMV antigen and/or RNA was found in lung, intestine, spleen, and lymph nodes, which corresponds with the subacute systemic phase of morbillivirus infection in terrestrial species and in marine mammals [256, 257]. The marked lymphoid depletion in spleen and lymph nodes is well known as the primary effect of morbillivirus infection, resulting in immuno- suppression and allowing facultative pathogens such

as *Bordetella bronchiseptica* to cause severe disease [66]. Surprisingly, case No. 2 had no pathological evidence of disease from such facultative pathogens. It is noteworthy that the infection was over its peak. This was suggested by the hyperplasia of lymphocytes in the prescapular lymph node and limited virus antigen expression in combination with lymphoid depletion. Again it is noteworthy that observed antibody titers were low despite the suspected duration of infection of several weeks based on the above observations. In seals, a maximum antibody titer is developed 3 weeks after infection despite lymphoid depletion [258].

The potential relationship between DMV infection and live stranding in these 2 white-beaked dolphins is speculative. In case No. 1, the CNS infection may have affected feeding behavior (resulting in emaciation) and/or ability to navigate and orientate. In case No. 2, the systemic infection may have caused malaise, resulting in separation from the mother and the rest of the social group, and subsequently emaciation and disorientation. In both cases, these effects may have led to stranding.

These 2 cases, as well as previous cases of DMV infection in white-beaked dolphins, raise questions about the role of white-beaked dolphins in the epidemiology of DMV and about the virulence of DMV for white-beaked dolphins. Regarding epidemiology, our data, together with the previously reported individual strandings of white-beaked dolphins infected with DMV in the Netherlands in 1990 [74] and on the North Sea coast of Germany in 2007, [73] indicate that DMV is recurrently present in the UK–North Sea population of white-beaked dolphins. Furthermore, phylogenetic analysis (Fig. 5) shows that the 3 DMV isolates from white-beaked dolphins in 2007 and 2011 had a common source, since they were more closely related to each other than to DMV isolates from other odontocetes, including striped dolphins in 2007 and 2011. Together, these findings suggest that white-beaked dolphins could be acting as a reservoir for this DMV. DMV observed in white-beaked dolphins in 2007 and 2011 is closely related to DMV observed in 1990. Morbilliviruses are highly contagious, cause infections of short duration, and induce long-lasting immunity in survivors. Therefore, reservoir populations for morbilliviruses need to be large [64]. Potentially, the North Norway-Barents Sea population, with up to 100 000 individuals, could fulfill this role. From there, DMV might spread recurrently to the smaller UK–North Sea population of 22 000 individuals. We observed a high incidence of morbillivirus infection in a small sample from this population. Regular infection of the UK–North Sea population may induce immune protection and prevent large-scale epidemics.

Regarding virulence of DMV for white-beaked dolphins, there is no evidence that these 2 DMV cases were representatives of a major DMV epidemic in white-beaked dolphins. First, there were 6 months between the dates of stranding of case No. 1, which had a chronic infection, and case No. 2, which had a subacute infection. Second, 2 other white-beaked dolphins that stranded on the coasts of the Netherlands and Belgium in winter 2011–2012 (case Nos. 3 and 4) were not infected by DMV. Third, there was no reported increase of white-beaked dolphin strandings on the coasts of the Netherlands, Belgium,

and Schleswig-Holstein, Germany, in 2011 (n= 5) or 2012 (n= 3) [259, 260] (U. Siebert, personal communication, 2013). Furthermore, DMV infection was not diagnosed as the proximate cause of death in either of the 2 white-beaked dolphin cases reported here. Together, these findings suggest that DMV is less virulent for white-beaked dolphins than, for example, for striped dolphins, in which major die- offs from DMV occurred in 1990 and 2007 [68, 70]. Species variation in susceptibility to morbillivirus infections is well known. For example, CDV is known to be more virulent for ferrets than for domestic dogs [261], and phocine distemper virus is known to be more virulent for harbor seals than for gray seals [66].

In conclusion, we have demonstrated 2 forms of DMV infection in white-beaked dolphins: subacute systemic and persistent neural. The combined data on individual strandings of DMV-infected white-beaked dolphins on the coasts of the Netherlands and Germany indicate that the UK–North Sea population is recurrently infected with DMV without undergoing a major die-off and suggest that DMV is not highly virulent for white-beaked dolphins. There is a need for further research on DMV in this species to further clarify its epidemiology and virulence. Rehabilitation centers should be aware of the risk of introducing morbillivirus into their center when admitting white-beaked dolphins. Upon autopsy of stranded white-beaked dolphins, special attention should be paid to analysis for morbillivirus infection in multiple organs, including the CNS. The relationship between cause of death and postmortem evidence of a morbillivirus infection in incidental mortalities of odontocetes should be considered cautiously.

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Nursing gray seal, Danish coast
Jonas Teilmann

7

INDICATIONS FOR BOTH HOST-SPECIFIC AND INTRODUCED GENOTYPES OF *STAPHYLOCOCCUS AUREUS* IN MARINE MAMMALS

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ABSTRACT

Staphylococcus aureus is present in the marine environment and causes disease in marine mammals. To determine whether marine mammals are colonized by host-specific strains or by strains originating from other species, we performed multi-locus sequence typing on ten *S. aureus* strains isolated from marine mammals in the U.K., the Netherlands, and the Antarctic. Four new sequence types of *S. aureus* were discovered. *S. aureus* strains from a southern elephant seal (n = 1) and harbor porpoises (n = 2) did not cluster with known *S. aureus* strains, suggesting that they may be host species-specific. In contrast, *S. aureus* strains from harbor seals (n = 3), other harbor porpoises (n = 3), and a grey seal (n = 1) clustered with *S. aureus* strains previously isolated from domestic ruminants, humans, or birds, suggesting that these *S. aureus* strains in marine mammals were introduced from terrestrial species.

INTRODUCTION

Staphylococcus aureus is an important pathogen for humans, domestic animals, and wildlife. On the one hand, *S. aureus* strains have co-evolved with their hosts and have therefore become host species specific, for example in cattle, pigs, horses and several other species [262-265]. On the other hand, *S. aureus* strains are not exclusively linked with their host species and cross contamination to other species occurs [266, 267].

Marine mammals are known to be infected with *S. aureus* [268-272]. However, these *S. aureus* isolates have not been characterized and therefore their origin is not known. In principle, *S. aureus* in marine mammals could have two origins. Marine mammals may be infected or colonized by *S. aureus* strains that have co-evolved with their host and are host species-specific. Alternatively, marine mammals may be infected with *S. aureus* strains originating from other host species.

The goal of this study was to determine whether marine mammals carry their own host species-specific *S. aureus* strains. Strains were therefore characterized by multi- locus sequence typing (MLST). The spread of *S. aureus* between species can be inferred from the host species of observed sequence type and the clonal complexes they belong to [273]. Therefore, we determined the sequence type of 10 *S. aureus* isolates obtained from various species of marine mammals from the Netherlands, the U.K., and the Antarctic, and compared them with known sequence types from other species in the dedicated S. aureus MLST eBurstv3 database (hosted by the Imperial College in London; www.mlst.net).

MATERIALS AND METHODS

Isolates of *S. aureus* from tissues or swabs of marine mammals were obtained and identified by use of standard methods [274]. Identification of *S. aureus* was confirmed by DNA probe test Accuprobe1 (Gen Probe, San Diego, CA 92121 USA). Tissues were obtained at necropsy from marine mammals in captivity or stranded in the Netherlands or the U.K. Three swabs from nose or mouth were obtained from apparently healthy marine mammals under rehabilitation in the U.K., as part of veterinary treatment or trapped in the Antarctic (Table 1). Sampling of the southern elephant seal was done in 1993. The sample was plated on MacConkey agar and Columbia agar and growth was freeze dried until 2000 when further processing occurred.

In two animals *S. aureus* infection was held responsible for their death (M1472/93/1 and M192/06/01). In two animals *S. aureus* caused a generalized bacterial infection (M218/03/1 and M148/00/1). In one harbor porpoise *S. aureus* infection caused a chronic retroperitoneal abscess (M1814/92/1) and in a grey seal *S. aureus* was found with other bacterial species in a very severe mouth ulcer with involvement of the underlying bone (M2122/93/2). In one harbor porpoise *S. aureus* was observed as a secondary infection together with severe pulmonary parasitism (M52/04/1) (Table 1).

S. aureus MLST was performed using standard DNA amplification and sequencing technology as described by Enright et al. [87]. Seven housekeeping genes were partially sequenced and sequence heterogeneity was assessed. All strains were assigned to a known sequence type number or—if new—given a new number by use of the previously mentioned *S. aureus* MLST database. Strains also were assigned to a clonal complex, defined as a group of sequence types that share six out of seven locus sequences and are assumed to have a common ancestor [275]. The MLST database was used to identify other species that had been infected with *S. aureus* strains of the same sequence type or clonal complex.

RESULTS AND DISCUSSION

Four of the ten *S. aureus* strains from marine mammals had sequence types not recorded before in the MLST database and were assigned new sequence type numbers: 1762 and 1766 from harbor porpoises (*Phocoena phocoena*), 1763 from a southern elephant seal (*Mirounga leonina*), and 1764 from a harbor seal (*Phoca vitulina*) (Table 1). The remaining six *S. aureus* strains had known sequence types that had been found previously in terrestrial birds and terrestrial mammals, including humans (Table 1).

The results of our study show a broad genetic diversity in the *S. aureus* strains isolated from marine mammals. The comparison of sequence types from the marine mammals in our study to known sequence types from other species suggest that some *S. aureus* strains

from marine mammals may be host species-specific, although more *S. aureus* strains from both marine mammals and other species need to be typed to be sure about this.

S. aureus strains from two harbor porpoises and a southern elephant seal may be host species-specific. The harbor porpoise sequence types 1762 and 1766 clustered with each other in a clonal complex, but not with any other sequence type in the MLST database (Table 1). The southern elephant seal sequence type 1763 did not cluster in a clonal complex with any other sequence type in the MLST database (Table 1). This suggests that these marine mammals have their own co-evolved variants of *S. aureus*, which are not closely related to *S. aureus* strains from terrestrial species.

The other *S. aureus* strains from marine mammals were sequence types that have previously been isolated from domestic ruminants (sequence types 1764, 130, and 630), humans (sequence types 47 and 22), or terrestrial birds (sequence type 692).

Sequence type 692, isolated from an oral lesion of a stranded grey seal, has previously only been isolated from birds (Table 1). Grey seals are known to predate on birds [276], which is a possible route of transmission in this case [277]. Predation on birds also is thought to be the route by which seals become infected with avian influenza viruses [278].

Sequence types 1764 and 130, isolated from harbor seals at rescue centres, clustered together in one clonal complex (Table 1). Sequence type 130 has previously been isolated from cattle and goats, and belongs to a clonal complex of sequence types isolated mostly from milk samples of cattle, goats, and sheep. Infection of harbor seals may have occurred due to contamination of sea water by agricultural runoff or due to infection by seal handlers who themselves carried *S. aureus* acquired from a ruminant source. A similar origin is suggested for sequence type 630 from a captive harbor porpoise: this sequence type has previously been isolated from dairy milk (Table 1).

Sequence types 47 and 22, isolated from stranded harbor porpoises, are *S. aureus* strains that until now have been frequently and exclusively reported in humans (Table 1). This suggests a human origin of these strains.

Contamination of coastal waters by feces from terrestrial species can be an important source of pathogens for marine mammals as is evident from the infection of sea otters (*Enhydra lutris nereis*) and bottlenose dolphins (*Tursiops truncatus*) with *Toxoplasma gondii*, a protozoan parasite whose oocytes are spread by cats [279, 280] *S. aureus* frequently colonizes the intestinal tract of healthy humans and terrestrial animals [281, 282] *S. aureus* is known to survive in sea water for at least 14 days [283]. Contamination of sea water by untreated sewage or agricultural runoff may therefore occur. Alternatively sea water contamination may be caused by recreational swimmers and bathers [284].

Parallel evolution of marine *S. aureus* strains leading to similar DNA sequences as found in terrestrial strains cannot be ruled out. Research on marine mammal populations that live outside the region where contamination of humans or agricultural strains is likely to occur may clarify if terrestrial sequence types do come from terrestrial contamination or are the products of parallel evolution of *S. aureus* strains in the marine environment.

Table 1 Characterization by multi-locus sequence type and clonal complex of *Staphylococcus aureus* isolates from marine mammals from the U.K., The Netherlands, and the Antarctic, and relationship to *S. aureus* isolates from humans and other host species.

Strain no.[a]	Host			Tissue sample		Multi-locus sequence type			Clonal complex	
	Species	Year and Location	Status	Location	Status	I.D. no.	No. of isolates	Other species infected	No. of sequence isolates	Other species infected (no. of isolates per species)
M2122/93/2	Grey seal	1993, Bodham Harbour, U.K.	Stranded	Mouth	Lesion	692	2	Chicken, Domestic pigeon	3	Pheasant, poultry, pigeon
M1472/93/1	Harbour seal	1993, Skye, U.K.	Rescue centre	Brain, meninges	Lesion	1764	1	None	8[b]	Cattle, goat, sheep not specified
M597/99/1 and M597/99/2	Harbour seal	1999, South Ronaldsey, U.K.	Rescue centre	Nose	Normal	130	2	Cattle, Goat	8[b]	Cattle, goat, sheep, not specified
A/G16/00/9	Southern elephant seal	2000, Gourlay Peninsula, Antarctica	Trapped	Mouth	Normal	1763	1	None	1	None
M1814/92/1	Harbour porpoise	1992, Nairn, U.K.	Stranded	Multiple[c]	Lesion	47	9	Human	21	Human (120), cattle (5), not specified (10)
M148/00/1	Harbour porpoise	2000, Blairmore, Dunoon, U.K.	Stranded	Multiple[d]	Lesion	1762	1	None	2[e]	None
M218/03/1	Harbour porpoise	2003, St. Coombs, Fraserburgh, U.K.	Stranded	Multiple[f]	Lesion	1776	1	None	2[e]	None
M52/04/1	Harbour porpoise	2004, East Beach, Lossiemouth, U.K.	Stranded	Lung	Lesion	22	100	Human	40	Human (136), goat (1), horse (1),
M192/06/1	Harbour porpoise	2006, Harderwijk, the Netherlands	Zoo	Multiple[g]	Lesion	630	3	Cattle (2), Not specified (1)	20	Human (268), cattle (39), not specified (23)

[a] Allocated by G. Foster, SAC Veterinary Services, Inverness, U.K.

[b] Same clonal complex.

[c] Retroperitoneal abscess, lymph node, spleen, small intestine.

[d] Brain, pericardial fluid, spleen, lung, liver, kidney, mesenteric lymph node, blood.

[e] Same clonal complex.

[f] Brain, liver, spleen, kidney, mesenteric lymph node.

[g] Heart, muscle, skin nodule, adrenal gland, subdermis, liver, kidney, cardiac lymph node, testis, lung, lung-associated lymph node.

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Elefant seals, Peninsula Valdes, Argentina

8

SUMMARIZING DISCUSSION

RELEVANCE OF THIS THESIS

The marine environment is significantly and increasingly impacted by human activities. These impacts cause an increase in morbidity and mortality which has resulted in a dramatic loss of biodiversity in the marine environment [1, 132]. Plastic waste, chemical pollution, overfishing, acidification and climate change are examples of how human activities influence and have influenced the marine environment. Many of these changes are long-term changes, like the introduction of PCBs and plastics, or will increase in impact during the next decades as is the case for climate change and acidification.

In this thesis I explored causes of morbidity and mortality in marine mammals with special attention for the impact of human influences. Changes in morbidity and mortality can of course also occur due to causes not related to human activities. Examples are the introduction of new pathogens or known pathogens into immune-naïve populations, changes in food availability and changes in predation habits of species, although also for these examples human activities may have played a role.

Knowledge of causes of morbidity and mortality, derived from autopsies, is valuable for three reasons. First, it provides an opportunity to evaluate effects of changes in the environment, although the etiology of disease is often multi-factorial and the effects of environmental changes may be difficult to discern. Nevertheless, information from autopsies in stranded marine mammals is often the only source of information available. Second, in combination with census reports, it can help to select which measures are appropriate for conservation efforts. Census reports can tell us whether populations are vulnerable. Subsequently, health assessments can tell us why populations are vulnerable and what can be done to help them. Health assessments can be based on samples from stranded, bycaught or hunted animals, and from catch-and-release programs. In the Dutch coastal region, stranded animals provide the sole source of sampling. The third and final reason for doing health assessments on marine mammals is that they may provide an early warning of negative impact by human activities on the marine ecosystem. Marine mammals are good sentinels for ecosystem health for several reasons. First, marine mammals mostly feed at high trophic level in the food chain, are long-lived, and have a blubber layer that effectively stores lipophilic pollutants. Biomagnification of toxic pollutants, like PCBs, occurs. This makes marine mammals more prone to negative effects of chemical pollution than other participants in the ecosystem. Second, cetaceans are dependent upon the use of sound for hunting and social communication. This group of animals is more sensitive to human-produced sound than the remainder of the ecosystem. Third, marine mammals are mostly fish feeders and therefore sensitive to competition with humans for food resources [285].

The contribution this thesis provides then is threefold. First, a description of specific diseases, which helps to make a diagnosis more easily and to understand the pathogenesis

and epidemiology better. Second, a description of the overview of causes of morbidity and mortality, which provides a baseline for comparison with the situation in the future. Third, findings which directly implicate a human influence.

OVERVIEW OF CAUSES OF MORBIDITY AND MORTALITY IN LIVE-STRANDED HARBOR PORPOISES

The foundation of this thesis was based on autopsies done on live-stranded harbor porpoises and a couple of white-beaked dolphins for the rehabilitation center SOS Dolfijn. The number of autopsies on harbor porpoises (61) was sufficient to make an overview of the main causes of stranding and mortality in live stranded harbor porpoises and further the knowledge on some of the more significant diseases of these animals. A significant disease was defined as a disease that was likely to have contributed to the stranding of the animal. Besides all the significant diseases, a large number of diseases were observed which had less severe impact and were considered incidental diseases. My overview focused on the significant diseases acquired in the wild, with less attention for incidental diseases and those diseases acquired during rehabilitation.
I compared my findings on causes of morbidity and mortality in live-stranded harbor porpoises with previous overviews of causes of morbidity and mortality in dead-stranded harbor porpoises. This raises the question whether a selection of live-stranded animals provides a similar sample of the population as a sample of dead-stranded animals. These samples would be different if certain diseases, or causes of mortality, would kill in a period too short for animals to be recovered alive from the beach. The most known and frequent cause of rapid death is bycatch. This was corrected for as much as possible. Another potential cause of rapid death in cetaceans is sepsis. However, the percentage of sepsis cases encountered in my investigation was completely in line with the percentage encountered in investigations on dead-stranded animals. There is no other indication of causes of mortality that kill in a short period. The assumption that the samples of dead- and live-stranded animals are similar seems therefore justified. Stranded animals are a sample of the diseased animals of a population that live close to shore. Results can thus not be extrapolated to the entire population as offshore animals may differ in gender and age class. Furthermore prevalence of disease in stranded animals gives no information about prevalence of disease in the entire population. With these limitations in mind one can look at the results of my investigation and compare them to the results of similar previous investigations.

The most frequently observed significant diagnosis was infectious pneumonia (30/61). In 19 animals a single infectious agent was identified and in 11 animals a combination of agents was observed. Three observations on pneumonia differed, or extended the

knowledge available from, previous similar harbor porpoise autopsy overviews [23, 24, 26, 286]. First, a higher prevalence of fungal pneumonias was noted than in previous reports. Second, bacterial pneumonias were observed that were not associated with, or the consequence of, a parasitic infection. Third, pneumonia was associated with infection with the presence of nematode species *Pseudalius inflexus*, *Torynurus convolutus* and *Halocercus* sp., but not with *Stenurus minor* infections. I also observed a significantly higher infestation with *Pseudalius inflexus* in adults compared to juveniles. The other nematode species were observed in equal prevalence and intensity in both age classes. Starvation was the second most frequent cause of stranding. Starvation was observed only in animals which were, based on their estimated ages, dependent upon their mothers or around weaning age. I found therefore no indications that lack of food was a cause for concern in harbor porpoises, based on autopsy results.

I observed more significant diseases in the brain, integument, liver and kidney than had been observed in previous research. Significant inflammation of the brain was observed in 7/61 animals. Inflammations were associated with fungal infections (n=3), viral infection (n=1) or of unknown etiology. Significant lesions of the integument were noted in in 7/61 animals. In 4 animals these lesions were caused by scavengers or predators. The other lesions were bacterial infections (2) and one combined viral and bacterial infection (1). Liver lesions consisted mostly of inflammations (5 out of 8). Only in one case it was clear which infectious organism was involved (*E. coli*). In the other cases no potential etiologic agent was found. Significant kidney lesions were observed in 5 out of 61 animals. These lesions had varying non-inflammatory etiologies.

In 5 animals a clear discrepancy was observed between severe clinical signs and mild or absent pathology on autopsie. In three animals overt nervous symptoms were not reflected by observed pathology in the central nervous system and in two animals kidney failure as was evident from clinical signs and clinical chemistry was not supported by gross pathology findings on autopsy.

Fungal infection was a significant diagnosis in 9 out of 61 animals. This was a much higher frequency than observed in previous harbor porpoise autopsy overviews [23, 24, 26, 286]. All fungal infections are likely to have been caused by the aspiration of infectious fungal conidia. In seven animals these conidia ended up in the lungs where they caused a fungal pneumonia. In the other two cases, the conidia settled in the middle ear where they caused otitis.

The majority of animals, 47 out of 61, died due to diseases of their organs and the majority of these animals, 28 out of 47, had lesions in multiple organs which accounted for their demise. The remaining animals were ten orphaned mother-dependent animals, of which seven were emaciated, and four for which the cause of stranding was not determined.

The most concerning finding in this thesis was the high prevalence of *Aspergillus* sp. infections in harbor porpoises (Chapter 2). This prevalence (15 %) was very much higher

than the prevalence observed in previous similar research projects (0-3 %) [22-24, 26]. *Aspergillus* sp. infections cause macroscopically obvious lesions which can be routinely diagnosed by culture and/or histology. It seems unlikely that previous investigations, which work according to similar protocols of sampling for histology and culture of gross lesions, would have missed these diagnoses. Increased exposure to *Aspergillus* sp. conidia (in birds), concurrent disease, lack of food, and impaired immunity (in birds and mammals) predispose for aspergillosis [163, 166, 287]. Compared to previous investigations, I found neither more starvation nor more concurrent disease, and there was no obvious reason to assume a higher level of exposure to *Aspergillus* sp. conidia. An increase in prevalence of fungal infections in harbor porpoises may therefore indicate that immunity was more impaired in the animals in my research compared to the animals in previous research in other regions of the North Sea. Toxic substances released by humans in the environment could have been responsible for this. Continuous frequent sampling of stranded harbor porpoises may clarify whether the increase in prevalence of fungal infections is a consistent finding and how prevalence changes with time and location. These changes should be compared with changes in concentration of pollutants to investigate whether correlations exist.

Brain lesions of unknown etiology were observed in 3/61 animals. In future, more extensive sampling of the brain and the application of new techniques like deep sequencing may shed more light on the etiology of this important site of disease in harbor porpoises. Imaging techniques, like MRI and CT may direct histological sampling and increase the chance of observing localized lesions, like those caused by herpesvirus infections. Pneumonia was the most frequently observed significant diagnosis in the investigated harbor porpoises. A question that remained is which lesions are indicative of a subclinical infection and which lesions are representative of a more serious clinical infection. Upon autopsy this is not always clear, partly because harbor porpoise lungs are difficult to evaluate. There may be several reasons for this. First, palpation is hampered by the cartilaginous structure of the bronchi and bronchioli. Second, changes in color due to inflammation are more difficult to identify in harbor propoises than in terrestrial animals or seals. Third, most harbor porpoises are infected with several species of lungworm as juveniles, and remain infected with a fairly high intensity throughout life [31]. Observations done on bycaught animals in Norway and Iceland illustrate that externally healthy animals have lung lesions which may have resulted in only subclinical effects. Bronchopneumonia was categorized as mild (35 %) or moderate (47 %) in these bycaught animals while most (82 %) were in good nutritional condition and had a concurrent lungworm infection (74 %) [29]. If we assume that bycaught animals in good nutritional condition are not significantly impacted by disease, then the lung lesions observed in these animals may be classified as incidental and subclinical. On the other hand, if we assume that the lesions in the lungs of dead or stranded animals, without serious lesions in other organs, have caused or contributed to their death or stranding, then these lung lesions may be classified as significant and

clinically relevant. It should be investigated whether the difference between significant and incidental lesions can be attributed to a difference in etiologic agents or inflammatory responses, or is due to the lung volume percentages involved in the infections. Modern imaging techniques like computed tomography scans may help to quantify the volume of lungs involved in an infection. In this way we may differentiate clinical from subclinical infections based on the etiologic agents involved or on the inflammatory response or quantity of lung volume affected or combinations thereof.

This and previous investigations provide a baseline overview of causes of morbidity and mortality of harbor porpoises in the North Sea and adjacent waters. Future autopsy projects should focus on changes in severity of disease, prevalence, and incidence of causes of morbidity and mortality. These changes may be caused by the pathogen (emergence of new diseases, introduction of known diseases in immune-naïve populations, increased virulence) the host (decreased immune resistance or decreased nutritional condition) or as a consequence of changes in the environment, which more often than not result from human activities (pollution, decrease in food resources). Comparisons with previous published similar research is crucial to note such changes. Comparisons can only be made successfully if methods and reporting are standardized. Autopsies are mostly done according to the protocol written by Kuiken and Baker with slight individual adjustments [23, 24, 26, 89, 286]. Reporting is unfortunately less standardized. Some suggestions I would like to make for future reporting are:

- Clarify which of the observed lesions are significant and which are incidental. Harbor porpoises usually have multiple lesions and the reader is poorly situated to judge which lesions are significant and which are incidental. A suggested pragmatic criterion is that a lesion is significant if it is considered to have contributed to the stranding or death of an animal. For bycaught animals such a criterion might be if a lesion is responsible for a diminished nutritional condition.
- Identify the cause of disease, if possible. Mere morphological diagnoses have a limited value, especially if one tries to compare the results of multiple investigations.
- Report results for autopsies for one species only.
- Report pneumonias separately for the different age classes, juveniles and adults. Pneumonia can be related to infection with *Pseudalius inflexus*. This infection differs in prevalence and intensity between juveniles and adults (this thesis, chapter 2). Prevalence and severity of pneumonia therefore has a relation with age class and cannot be compared unless reported separately for each age class.
- Report evaluations of lesions or parasitic infestations , as far as possible, according to objective criteria. This is not always easy, especially in the case of pneumonias. For pneumonias, experts should develop generally accepted criteria for what is a mild, moderate or severe pneumonia. For parasitic infections an estimate of the number of parasites involved is easily provided and useful if comparisons between different publications have to be made.

I had the opportunity to investigate five important pathogens of marine mammals more closely: pestivirus, herpesvirus, morbillivirus, lungworms and *S. aureus*.

Pestiviruses

I had observed three cases of encephalitis with clinical symptoms of central nervous disease and confirmation of encephalitis by histology without being able to identify a cause of the encephalitis. I got the opportunity to have samples of these animals investigated by next generation sequencing (NGS) to see if an etiologic agent could be found. A novel pestivirus, tentatively named PhoPeV was discovered. This virus had caused systemic infections in 9 % of the 112 animals investigated [44] but apparently was not associated with the observed brain lesions.

The possibility that this virus does do harm remains, although in our samples no lesions associated with PhoPeV infection were observed. Variation in virulence between different genotypes, reproductive failure, immunosuppression and pestivirus jumping from one species to another are all in potential harmful and not contradicted by our investigation. We found two tentative genotypes of pestivirus in our sample, showing that in harbour porpoises pestivirus has genetic variation which underscores the possibility of the existence of more virulent genotypes. Reproductive failure leading to resorption, abortion or the birth of non-viable offspring is unlikely to be detected by investigating stranded animals and thus could be associated with the pestivirus infections we observed. Immune suppression can be related to suppression of subsets of lymphocytes or intervention with interferon production [288-290]. Such changes may not be detected during autopsy or histologic inspection of samples. Finally pestivirus readily jumps from one species to another in terrestrial species with differing virulence in different species [48]. A jump of the virus from one aquatic species to another may thus be associated with significant disease and thereby be a threat to small fragmented populations as may have speculatively occurred with the Pyrenean chamois [53]. For morbillivirus there are strong indications such a jump has occurred from long-finned pilot whales to striped dolphins, which underscores the possibility of viruses jumping from one species to another in cetaceans. All the mentioned possibilities of potential pathology (occurrence of more virulent isolates, reproductive failure, immunosuppression and infection from one species by another) warrant further research to investigate whether pestivirus has a deleterious effect on marine mammal health.

This research shows how novel techniques can be applied successfully to archived samples, leading to discovery of novel infectious agents. The importance of keeping banks of archived tissues for the application of novel technologies is thereby underscored. Finally, since the cause of the encephalitis observed in the initial three porpoises studied by NGS has not been clarified, studies into its cause should still be continued.

Herpesvirus

Herpesvirus infection may cause a potentially fatal disease in cetaceans and the severity of a herpesvirus infection is associated with immune status of the host [55, 209]. Little is known about herpesvirus infection in harbor porpoises and basic knowledge on different viral species, virulence and prevalence are largely lacking. The relationship between herpesvirus virulence and immune status is relevant in view of the concerns over the immune status of harbor porpoises due to a possible negative influence of chemical pollutants. Monitoring severity of herpesvirus-associated disease may provide an instrument to evaluate negative influences of these pollutants on the immune status of harbor porpoises.

I found evidence for three different herpesviruses in harbor porpoises, one gamma- and two alphaherpesviruses. Prevalence of infection with these virus species was less than 5 %. Only in one animal (2 % of the sample) significant disease was observed: severe encephalitis, caused by alphaherpesvirus infection, PPHV2. An incidental lesion was a genital plaque in another animal (1 % of sampled animals), caused by a gammaherpesvirus infection. Thus I have provided a baseline of herpesvirus infections in harbor porpoises in our region. A baseline characterized by low prevalence of clinically manifest herpesvirus-associated disease. This baseline can be used in monitoring changes in prevalence of clinically manifest herpesvirus infections and severity of associated disease in harbor porpoises in future studies.

Genital herpesvirus infection in bottlenose dolphins was investigated for the same reasons as herpesvirus infections in harbor porpoises. Here the perspective was to provide a baseline of a population of animals in excellent nutritional condition and not exposed to chemical or noise pollution as reference for their wild counterparts, which at times are more exposed natural and anthropogenic stressors. The successful *in vitro* propagation of the herpesvirus involved will facilitate additional investigation into the epidemiology of this herpesvirus possible based on antibody detection.

Mucosal plaques on the male genitals and in the female genital slit of a group of bottlenose dolphins were associated with a gammaherpesvirus infection. These bottlenose dolphins lived in a zoological collection which was endemically infected. Age of infection, based on seroconversion, was around the start of copulation behavior (from a few weeks onwards in males, and from 4-5 years onwards in females). The infection with genital herpesvirus was not associated with abortion or urogenital neoplasia. The DNA sequence of the herpesvirus found was 100 % identical to that of a genital herpesvirus isolated from bottlenose dolphins that had stranded in the region where the founding fathers of this collection had been caught and was less similar (91 % identity) to herpesvirus DNA sequences observed in bottlenose dolphins that had stranded in an adjacent but different

region (Gulf of Mexico, Caribbean vs West Atlantic Ocean waters).

The knowledge coming from my research on genital herpesvirus in bottlenose dolphins can be used in health assessment programs in catch-and-release operations which take place for example in the US [291-293] or in autopsy programs on stranded animals. These regular operations are partly conducted in heavily polluted areas where the health of the animals is compromised by pollution, like in Barataria Bay which was heavily hit by the oil spill from the Deep-Water Horizon [293, 294]. It would be of interest to investigate whether prevalence of genital herpesvirus infection and the severity of associated lesions is different in this population of bottlenose dolphins compared to populations under human care or from less polluted waters.

Morbilliviruses

Morbilliviruses are well-known pathogens of marine mammals with a history of causing large scale epidemics in immune naïve populations of harbor seals, striped dolphins and bottlenose dolphins [66]. Transmission of morbilliviruses in the aquatic-environment is only partly understood as is the clinical course and relevance of morbillivirus for different cetacean species [79]. Two cases of dolphin morbillivirus (DMV) infection in white-beaked dolphins were therefore subject of further investigation.

DMV infection was observed in two white-beaked dolphins, an adult with chronic neurological infection and a calf with subacute systemic infection. Histology and clinical observations gave different assessments of the consequences of the lesions. For the systemic case it was speculated that the disease caused malaise which lead to separation of calf and mother, resulting in starvation and weakening of the calf. In the neurological case, lesions may have caused a sporadic tremor and a decrease in immune resistance which necessitated continuous medication with antibiotics to ward off secondary infections.
DMV in the white-beaked dolphins did not appear to be highly pathogenic considering the following circumstantial observations: it was detected four times in stranded white-beaked dolphins on the shores of the North Sea, in 1990, 2007 and twice in 2011, without causing massive die offs, and it was not the proximate cause of death in either of the cases seen by me. DMV was diagnosed in three out of 25 autopsies done on white-beaked dolphins which had stranded on the Dutch, German and Belgium coasts between 2000 and 2012. This is a relatively high frequency, for example in comparison with harbor porpoises. In the latter species, it was diagnosed only once in the 278 animals autopsied between 1990 and 2016 [22-24].
Analysis of DMV genetics and white-beaked dolphin population structure indicated DMV could be endemic in the northern population, which lives in the North Norway-Barents

Sea from where it regularly spreads to the southern population, which lives around the UK and in the North Sea. The DMV observed in white-beaked dolphins was closely related to the DMV observed in the striped dolphin DMV outbreak in the Mediterranean in 1990 but not to the DMV observed in the outbreak amongst striped dolphins in the Mediterranean of 2007 (chapter 6).
In conclusion DMV occurred at a surprisingly high prevalence in the population of white-beaked dolphins in the North Sea while the associated disease symptoms were less severe than noticed during outbreaks of morbillivirus in harbor seals, striped dolphins and bottlenose dolphins.

Lungworms

The main findings on lungworm infection-the pathogenicity of the different species encountered, and the relationship between bacterial pneumonia and lungworm infection-have been discussed above (see page 161, 163-165). One striking feature of lungworm infections in harbor porpoises is that prevalence and intensity of infection do not decrease with age. We even observed an increase in prevalence and intensity for *P. inflexus* in adult animals compared to juveniles. It seems parasite and host find a balance where the immune system does not repel adult parasites and intense infections do not cause significant damage to the host. Donkeys and hedgehogs are also known for carrying large amounts of lungworms when adult. In these species, however, an inflammatory reaction is observed [295, 296]. Inflammatory reactions in harbor porpoises are mostly associated with larval infections of lungworms [22, 297]. One may speculate whether parasitic pneumonias are caused by an inflammatory reaction towards larvae or are the result of an over-reaction of the immune system toward adult nematodes.

Staphylococcus aureus

S. aureus infection in marine mammals was investigated because this bacterium is both a well-known and important pathogen of terrestrial mammals, including humans, and of marine mammals [83, 84, 298]. I speculated whether contamination of coastal waters with *S. aureus* strains by humans or their livestock could have been the source of marine mammal infection.

I found new strains, unrelated to previously observed strains, in an Antarctic elephant seal living far away from potential terrestrial contamination and in two harbor porpoises stranded on the UK coasts (actually on either side of the main island). One strain in a gray seal was also observed in birds, two strains in harbor porpoises had previously exclusively been seen in humans, three strains isolated from two harbor seals and a harbor porpoise in rehabilitation centers were otherwise mostly observed in dairy cattle, goats and sheep. These findings suggest that the *S. aureus* strains were either host-specific or

came from contamination by humans (e.g. swimmers and sewage) or food animals (e.g. contamination by run-off of manure into the sea or via human handlers).

Further sampling and typing of pathogenic *S. aureus* can indicate to what extent terrestrial contamination of the aquatic environment poses a risk to marine mammals. A remaining question is whether parallel evolution has created similar sequences in the marine environment as in the terrestrial environment or whether strains with terrestrial sequences are found as a consequence of contamination from terrestrial sources. Research on marine mammal populations that live outside the region, where contamination by humans or food animal strains is less likely to occur, may give an answer to this question.

AREAS OF FUTURE RESEARCH INTO PATHOLOGY OF MARINE MAMMALS

The two reasons for me to do research into marine mammal pathology were curiosity, and concern for the conservation of marine mammal species. Diseases can threaten marine mammal species. New emerging infections, increased virulence of pathogens, and changes in population structure as well as the environment may cause diseases to become more severe or prevalent [299] .

The most important changes in the environment are the result of human activities [1] and this is why the study of disease can be informative on the impact of humans on the environment. One of the human impacts which is a continuing concern is the potentially negative influence of persistent organic pollutants on the immunocompetence of marine mammals [77, 104, 146, 167, 300]. If we wish to notice subtle changes in disease prevalence and severity, such as those that may potentially be caused by chemical pollution, then regular surveys on causes of morbidity and mortality on an international scale are needed. Herpesviruses, lungworms and *Aspergillus fumigatus* deserve special attention as potentially good sentinels for two reasons. First, hosts experience continuous exposure to lungworms and *Aspergillus* infections (not certain for herpesvirus) and second, in other species severity of disease caused by these pathogens is negatively related to immunocompetence of the host [23, 163, 166, 225, 297, 301].

Basic knowledge on prevalence, severity of disease, and disease transmission is rudimentary in each of the pathogens (pestivirus, herpesvirus, morbillivirus, *S. aureus* and lungworms) I studied. Individual pathogens should be studied further to fill these gaps in our knowledge. I refer to the different chapters of this thesis and the above discussion for the detailed recommendations I could make based on my research.

The main sources for material of cetacean populations are stranded animals. However, catch-and-release programs with the aim of health assessment are executed in few places [302] and may be considered in our region in future. Furthermore, bycaught animals

deserve more attention than they get at present as they provide a valuable sample of the healthy population. Coordination of research efforts by an international organization could improve the effectivity and safeguard the continuation of such investigations, especially if international agreements by governments could be realized which could secure the necessary funds.

With the commitment of sufficient resources, research data will lead to the most sensible advice concerning conservation of our marine mammals and their habitat.

Waddenzee

9

NEDERLANDSE SAMENVATTING

INLEIDING

Het dierlijk leven in zee is in de afgelopen veertig jaar verminderd met 50 %. Deze catastrofe wordt vrijwel volledig veroorzaakt door de gevolgen van menselijk handelen. Het is daarom noodzakelijk inzicht te verwerven in welk menselijk handelen de meest nadelige invloed heeft op het zeemilieu. Onderzoek naar ziekten en ziekteverloop kan behulpzaam zijn om dit inzicht te verkrijgen. Ziekte is immers de uitkomst van de interactie tussen gastheer, ziekteverwekker en milieu.

Het bestuderen van ziekten bij zeezoogdieren is in dit verband extra waardevol, omdat zeezoogdieren een signaalfunctie kunnen vervullen. Zeezoogdieren zijn buitengewoon gevoelig voor de meest voorkomende menselijke invloeden zoals daar zijn: bijvangst in visserij, chemische en akoestische vervuiling en uitputting van visbestanden. Het zeezoogdier is als de spreekwoordelijke kanarie in de kolenmijn. Los daarvan is het voor het in stand houden van populaties zeezoogdieren ook van belang om ziekten en doodsoorzaken te kennen, bijvoorbeeld nieuwe en mogelijk bedreigende infectieziekten.

Het opvangcentrum voor gestrande kleine tandwalvissen, SOS Dolfijn, bood jarenlang een unieke mogelijkheid om ziekten bij deze zeezoogdieren te bestuderen. SOS Dolfijn is een stichting die van 2003 tot 2016 gehuisvest was op het terrein van het Dolfinarium in Harderwijk. Het opvangcentrum ving in die tijd kleine gestrande tandwalvissen-voornamelijk bruinvissen- op met het doel om ze na genezing weer uit te zetten in de Noordzee. Dieren die onverhoopt kwamen te overlijden, werden voor sectie aangeboden aan de afdeling Viroscience van het Erasmus Universitair Medisch Centrum te Rotterdam. Door deze samenwerking kon ziekte zowel bij het levende dier als postmortaal bestudeerd worden, wat voor in het wild levende tandwalvissen uniek is.

Het onderzoek naar ziekten van in het wild levende tandwalvissen staat nog in de kinderschoenen. Er is weinig precieze kennis over welke ziekteverwekkers belangrijk zijn, laat staan hoe vaak en op welke wijze ze ziekte en dood bij tandwalvissen veroorzaken. Toch is deze precieze kennis van evident belang als we de invloed van menselijk handelen op ziekte en ziekteverloop in kaart willen brengen. De invloed van menselijk handelen kan zich namelijk op subtiele wijze uiten terwijl ze tegelijkertijd bedreigend kan zijn.

Op twee manieren is onderzoek gedaan. Ten eerste is er een overzicht gemaakt van ziekten en strandingsoorzaken bij levend gestrande bruinvissen op onze Noordzeekust. Ten tweede zijn individuele ziekteverwekkers nader onderzocht. Een ziekteverwekker kwam in aanmerking voor onderzoek als reeds bekend was dat deze ziekte veroorzaakte bij zeezoogdieren of als bekend was dat de ziekteverwekker ziekte veroorzaakte bij andere diersoorten en er nog geen onderzoek naar deze ziekteverwekker bij zeezoogdieren was verricht.

OVERZICHT VAN ZIEKTE EN STRANDINGSOORZAKEN BIJ LEVEND GESTRANDE BRUINVISSEN

Eenenzestig bruinvissen zijn onderzocht in de periode van 2003 tot 2016. Alle dieren zijn volgens een vast protocol ontleed en orgaanmonsters zijn microscopisch beoordeeld. Indien het eerste onderzoek daar aanleiding toe gaf zijn vervolgonderzoeken ingesteld, zoals bacteriologisch, virologisch of immunohistochemisch onderzoek en/of elektronenmicroscopie. Voor iedere bruinvis is vastgesteld welke de vermoedelijke oorzaken van stranding waren. Strandingsoorzaken zijn vergeleken met doodsoorzaken van bruinvissen uit andere regio's van de Noordzee (België, Duitsland, Verenigd Koninkrijk) zoals gevonden in eerdere onderzoeken.

Overeenkomstig deze onderzoeken behoorden longontsteking en verhongering tot de meest frequente strandingsoorzaken. In alle onderzoeken is verhongering vastgesteld bij de nog zogende bruinvissen. Waarschijnlijk verhongerden deze dieren doordat ze om onbekende redenen te vroeg van hun moeder gescheiden waren.

In tegenstelling tot de eerder gedane onderzoeken, die bij de meeste dieren een enkele doodsoorzaak aanwezen, kwamen wij tot de conclusie dat stranding bij de helft van de onderzochte dieren niet door één maar door meerdere aandoeningen werd veroorzaakt.

Er is controverse over de mate waarin zware infecties met parasieten in de longen dodelijk zijn dan wel een vereiste zijn voor het optreden van bacteriële pneumonie. Door een getalsmatige analyse van de wormbesmetting hebben we vastgesteld dat een zware infectie met longwormen geen ernstige gevolgen voor de gezondheid hoeft te hebben en een bacteriële pneumonie onafhankelijk van een longwormbesmetting kan voorkomen.

De meest verontrustende observatie was een veel hogere prevalentie van schimmelinfecties in dit onderzoek in vergelijking met eerdere onderzoeken (15 % versus 2-3 %i). Het betreft hier schimmelinfecties die ernstig genoeg waren om stranding of dood te veroorzaken. Deze observatie is verontrustend, omdat de meest voorkomende oorzaak van een bedreigende schimmelinfectie bij zoogdieren een gebrekkige weerstand is. Mogelijk was de weerstand van de groep bruinvissen die wij onderzochten aangetast.

Ons onderzoek kan als ijkpunt dienen voor vervolgonderzoeken om veranderingen in ziekten en doodsoorzaken bij bruinvissen vast te stellen. Zulke veranderingen kunnen dan gerelateerd worden aan veranderingen bij de bruinvis, de ziekteverwekkers of het milieu.

ONDERZOEK NAAR INDIVIDUELE ZIEKTEVERWEKKERS

Pestivirus bij bruinvissen

Pestivirussen zijn met name bekend als belangrijke ziekteverwekkers bij varkens en koeien. Gedurende een zoektocht naar de veroorzaker van hersenontsteking bij bruinvissen werd een pestivirus in de hersenen aangetroffen met behulp van metagenomics. Metagenomics is een techniek waarbij al het genetisch materiaal in een monster wordt onderzocht en vervolgens vergeleken wordt met alle bekende genetische codes. Vervolgonderzoek in de overige weefsels van de betrokken bruinvissen toonde aan dat de infectie in alle organen gevonden kon worden. De infectie veroorzaakte geen ziekteverschijnselen. De vondst van pestivirus in de hersenen was dus een toevalsbevinding en hield geen verband met de hersenontsteking.
Van 113 bruinvissen werden meerdere weefselmonsters nagekeken. Negen procent van de dieren bleek besmet te zijn met pestivirus. Wederom kon het virus niet gerelateerd worden aan ziekteverschijnselen. Het virus miste een eiwit dat in alle andere bekende pestivirussen wel gevonden wordt en wat het virus helpt het immuunsysteem van de gastheer te omzeilen. Hoewel het virus vooralsnog geen ziekte lijkt te veroorzaken bij bruinvissen, is nader onderzoek nodig om te bepalen of dat altijd zo is. Mogelijk veroorzaakt het virus wel ziekte in bruinvispopulaties die nog nooit eerder met het virus in aanraking zijn geweest.

Herpesvirusinfecties bij bruinvissen

Herpesvirusinfecties kunnen bij tandwalvissen milde aandoeningen van huid en genitalien veroorzaken, maar ook dodelijk verlopen als gegeneraliseerde infectie of als infectie van de hersenen. Bij één van de ontlede bruinvissen werd een door herpesvirus veroorzaakte hersenontsteking gediagnosticeerd. De diagnose werd onderbouwd door het aantreffen van typische insluitlichaampjes in de celkernen van de hersenen en ontstekingsreactie rondom deze celkernen. Bovendien werden herpesvirusachtige deeltjes gevonden in de insluitlichaampjes en werd herpesvirus DNA geïsoleerd uit de hersenen van deze bruinvis.
Bij een ander dier werd een herpesvirusinfectie van de genitaliën gediagnosticeerd. Een gladde, bleke plakkaatvormige verdikking van de genitale slijmvliezen was zichtbaar. De diagnose werd gebaseerd op insluitlichaampjes in de celkernen ter plekke van de infectie, herpesvirusachtige deeltjes in deze insluitlichaampjes en het aankleuren van herpesvirus RNA produkt in de cellen van plakkaatvormige verdikking.
Beide bruinvissen hadden een verschillend herpesvirus.
Naar aanleiding van deze bevindingen werden opgeslagen swabs van mond-, genitaal- en blaasgat- slijmvlies (afgenomen bij binnenkomst van een bruinvis bij SOS Dolfijn) en ingevroren hersenmonsters van ontlede dieren retrospectief onderzocht op de aanwezigheid van herpesvirus DNA. Drie verschillende herpesvirussen werden aangetoond: één

herpesvirus welke verantwoordelijk was voor de genitale plak, een tweede herpesvirus welke verantwoordelijk was voor de hierboven beschreven hersenontsteking en een derde herpesvirus welke werd aangetroffen in diverse organen en swabs van in totaal zes dieren. Het laatste herpesvirus kon niet in verband worden gebracht met aantoonbare ziekteverschijnselen. De prevalentie van de herpesvirussen varieerden van 1% (genitaalplak), 2 % (hersenontsteking) tot 5 % (herpesvirus zonder ziekteverschijnselen).
Herpesvirus bij bruinvissen kan dus een dodelijk verlopende infectie veroorzaken, maar wordt vooralsnog slechts in lage frequentie aangetroffen.

Genitale herpesvirus bij tuimelaars

Het aantreffen van herpesvirus in een genitale plak bij een bruinvis vormde aanleiding om de oorsprong van soortgelijke aandoeningen te onderzoeken in een collectie tuimelaars van een dierentuin. Tevens werd onderzocht of er aanwijzingen waren dat de bij tuimelaars aangetroffen herpesvirusinfectie, abortus en infecties bij pasgeborenen veroorzaakte of dat het tumoren veroorzaakte op de genitaliën. Laatstgenoemde effecten zijn bekend van herpesvirusinfecties bij andere diersoorten.
De diagnose herpesvirus als veroorzaker van de genitale plaks werd gesteld door het aantonen van herpesvirus DNA op slijmvliesmonsters uitsluitend van dieren met genitale plaks en niet van dieren zonder plaks. Het aantonen van herpesvirusachtige deeltjes in een biopt van een plak ondersteunde de diagnose.
We konden de epidemiologie van het virus in deze collectie tuimelaars onderzoeken door in gearchiveerde sera na te kijken wanneer dieren geïnfecteerd waren. Het bleek dat vrijwel alle dieren geïnfecteerd werden zodra ze copuleerden. Voor mannetjes was dat soms al op 1-jarige leeftijd en voor vrouwtjes vanaf 5-jarige leeftijd. Dit komt overeen met observaties van de leeftijd waarop copulaties voor het eerst worden gezien.
Om te kijken of er aanwijzingen waren of de herpesvirusinfectie andere ziekteverschijnselen veroorzaakte dan genitale plaks is gekeken naar de pathologische verslagen van overleden dieren uit de collectie. In deze verslagen werd geen melding gemaakt van genitale tumoren. In de geconserveerde organen van geaborteerde foetussen en pasgeboren overleden dieren is geen herpesvirus DNA aangetroffen.
Samengevat, genitaal herpesvirus was endemisch bij deze collectie dierentuintuimelaars en werd overgedragen door paargedrag. Er zijn geen aanwijzingen dat de herpesvirusinfectie meer ziekteverschijnselen veroorzaakt dan het optreden van onschuldige plaks op de genitale slijmvliezen.

Morbillivirus bij witsnuitdolfijnen

Morbillivirus is een beruchte ziekteverwekker bij zeezoogdieren. Bij gewone zeehonden, tuimelaar dolfijnen, gestreepte dolfijnen en gewone dolfijnen is het virus verantwoordelijk voor epidemieën met grootschalige sterfte. Aangenomen wordt dat deze epidemieën

worden veroorzaakt doordat het virus voor het eerst in een populatie wordt geïntroduceerd. Zo'n populatie heeft dan nog geen afweer tegen het virus en moet nog groepsimmuniteit ontwikkelen. Het is nog onbekend welke dieren (of diersoorten) dit virus in deze kwetsbare populaties hebben geïntroduceerd. Bij witsnuitdolfijnen, die regelmatig op de Nederlandse kust stranden, is het virus gevonden maar is het onbekend hoe ziekmakend het is.

Twee witsnuitdolfijnen, levend opgevangen door SOS Dolfijn, bleken na overlijden geïnfecteerd te zijn met morbillivirus. Het ziekmakend effect van het virus kon zo zowel bij leven als na overlijden onderzocht worden. Tevens zijn twee andere witsnuitdolfijnen, die in dezelfde periode dood strandden, onderzocht. Deze dieren bleken niet geïnfecteerd met morbillivirus.

De eerste levend opgevangen witsnuitdolfijn had een chronische morbillivirusinfectie van de hersenen. Het virus is aangetoond in de hersenen door middel van het aantonen van het genetisch materiaal van het virus en door het aankleuren van het virus zelf met behulp van specifieke antilichamen. De hersenen van de witsnuitdolfijn vertoonden opmerkelijke ontstekingsverschijnselen daar waar het virus aanwezig was. Deze witsnuitdolfijn, die reeds zes maanden in het opvangcentrum verbleef, had slechts zeer geringe zenuwverschijnselen en werd voorbereid om te worden uitgezet toen hij stierf. De doodsoorzaak had geen verband met morbillivirusinfectie maar was vermoedelijk een shockreactie op een toegediend medicijn.

De tweede levend opgevangen witsnuitdolfijn had een subacute longinfectie met het morbillivirus. Genetisch materiaal van het virus werd aangetoond in de longen en in enkele cellen van lymfknopen, milt en darmen door het aankleuren van het virus zelf met behulp van specifieke antilichamen. Sommige lymfknopen lieten een gebrek aan witte bloedcellen zien wat past bij de acute fase van een morbillivirusinfectie. Daarentegen liet een enkele lymfknoop sterke regeneratie van witte bloedcellen zien, wat past bij de herstelfase van de infectie. Vanwege ernstige vermagering en een gebrek aan weerstand werd dit dier daags na binnenkomst geëuthanaseerd.

Het aangetroffen morbillivirus is geanalyseerd en vergeleken met morbillivirussen die eerder waren gevonden bij andere soorten tandwalvissen en bij geïnfecteerde witsnuitdolfijnen, die gestrand waren in 2007 en 1990. Het morbillivirus bleek voornamelijk nauw verwant aan morbillivirus aangetroffen bij andere witsnuitdolfijnen en was waarschijnlijk geen bron van infectie voor andere tandwalvissoorten.

Samenvattend liet dit onderzoek zien dat een morbillivirusinfectie bij witsnuitdolfijnen in de Noordzee niet noodzakelijkerwijs tot sterfte en epidemieën leidt. Mogelijkerwijs is deze groep dieren beschermd door een regelmatige herinfectie vanuit de grote populatie witsnuitdolfijnen welke in de Noordelijke IJszee en Barentszzee leeft.

Staphylococcus aureus infectie bij meerdere zeezoogdiersoorten

Staphylococcus aureus is een bacterie die leeft als een onschuldige kostganger op de huid en slijmvliezen van warmbloedige dieren. Echter bij gelegenheid kan de bacterie ziekte verwekken. Dit gebeurt frequent. De bacteriesoort staat bekend als een belangrijke ziekteverwekker bij landzoogdieren en mensen. Twee bruinvissen in een dierentuin overleden aan een hartspierontsteking door *S. aureus*. Dit wierp de vraag op wat de oorsprong was van deze infectie met *S. aureus* en in bredere zin of zeezoogdieren besmet raken door soorteigen *S. aureus*-stammen of door stammen afkomstig van landzoogdieren. *S. aureus* is een bacterie die redelijk soortgebonden is maar makkelijk een overstap naar andere soorten kan maken.

De oorsprong van een *S. aureus*-stam kan achterhaald worden door genetische analyse van zeven huishoudgenen, zogeheten Multi Locus Sequence Typing (MLST). Negen *S. aureus* stammen verkregen uit monsters van zeezoogdieren (een zeeolifant, twee gewone zeehonden, een grijze zeehond en vijf bruinvissen) werden getypeerd. De stam gevonden bij de zeeolifant op Antarctica en twee bruinvissen op de kust van het Verenigd Koninkrijk hadden geen enkele relatie met eerder getypeerde *S. aureus*-stammen en waren hoogstwaarschijnlijk soorteigen stammen. Bij een grijze zeehond werd een *S. aureus*-stam gevonden die alleen bij vogels was aangetroffen. Waarschijnlijk is deze zeehond geïnfecteerd geraakt toen hij een vogel opat. Bij de overige dieren werden stammen aangetroffen die bekend zijn bij mensen of landbouwhuisdieren. Mogelijk zijn deze dieren geïnfecteerd geraakt door menselijke verzorgers in een opvangcentrum, menselijke verontreiniging door rioolafvoer of strandbezoekers, of door verontreiniging van het zeewater met dierlijke mest door mestafvoer richting zee via regen en rivierwater.

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ADDENDA

BIOGRAPHY

Cornelis Erik (Niels) van Elk was born on the 18th of May 1963 in Utrecht, the Netherlands. He finished high school at the Geert Groote College in Deventer in 1981. He obtained his Bachelor of Science in Marine Sciences (Marine Biology and Physical Oceanography) in 1984 from the University College of North Wales, Bangor. After his national service in the navy he started his study in veterinary science at the University of Utrecht in 1986. In 1994 he graduated with a focus on farm animal medicine and tropical veterinary medicine. After graduation he worked as a farm and companion animal clinician for five years. He continued his career at a marine mammal zoo in the Netherlands (Dolfinarium Harderwijk) from 1998 to 2017. During this period, he was responsible for the veterinary care of the zoo animal collection and a rehabilitation center for small toothed cetaceans. Beside his work as a clinical veterinarian he was responsible for the scientific program of the Dolfinarium Harderwijk, communication with media and politicians, and coordinated the breeding program of bottlenose dolphins in Europe. In 2017 he left the Dolfinarium and started his own company which offers consultation on conservation, veterinary care and welfare of display animals, and euthanasia of moribund large whales. He assisted in the last-ditch effort to save the vaquita (a small toothed cetacean) in the gulf of Cortes (Mexico) and participated in workshops on the conservation of threatened cetacean species. He works in a clinic for companion animal medicine beside his consultancy work. He would like to use his skills to help communicate the science behind conservation to the general public, media and politicians.

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Het voorstel

Het was in het voorjaar 2003 of 2004, toen ik onverwacht bezoek kreeg op het Dolfinarium. Ab Osterhaus en Thijs Kuiken kwamen met de vraag of ik geïnteresseerd was in het doen van een promotieonderzoek onder hun supervisie. Het was een win-win-win situatie voor het onderzoek, het Dolfinarium en voor mij persoonlijk. Het vertrouwen in mij was blind. Ik had op geen enkele wijze in mijn bestaan aanleiding gegeven voor enig vertrouwen in mijn wetenschappelijke capaciteiten. Voor het zien van deze mooie kans en het blinde vertrouwen in mij gesteld wil ik Ab en Thijs bedanken.

Support by my employer

The excellent proposition of Professor Osterhaus and Kuiken had to be presented to my management. Would they consider this research relevant and in the benefit of the Dolfinarium? Would this fit in their vision of what the purpose was of a marine mammal park? The management and owners at that time, Compagnie des Alpes, were positive about the proposition and provided me the opportunity to pursue this research. I would like to express my gratitude specifically towards Jan Reuvers. He has supported my research consistently throughout the years and assured the continuing support by Compagnie des Alpes. He realized as well how important this research was for my personal and professional development.

Onderzoeksmateriaal

Het materiaal wat ik gebruikte voor mijn onderzoek was afkomstig van dieren en data verkregen tijdens de opvang en verzorging van gestrande kleine tandwalvissen door SOS Dolfijn. SOS Dolfijn is een stichting die met raad en daad hulp verleent als kleine en grote walvissen stranden op de Nederlandse kust. Naast een heel leger van onvermoeibare en enthousiaste vrijwilligers zijn er vier vaste krachten die sinds jaar en dag de stichting draaiende hielden: Annemarie van den Berg, Jolanda Meerbeek, Bianca van 't Hul en Eligius Everaarts. Ik wil Eligius, Jolanda, Bianca en Annemarie bedanken voor alle steun en het verrichte werk ten behoeve van mijn promotieonderzoek. Jullie assistentie hield niet op toen de stichting zelf in zwaar weer kwam doordat nieuwe eigenaren van het Dolfinarium opvang en onderzoek niet wenste te steunen, zoals de voormalige eigenaren

dat wel hadden gedaan, waardoor de stichting zonder huisvesting kwam te zitten. Ik weet dat dit laatste op jullie persoonlijk een zware wissel heeft getrokken en bewonder jullie doorzettingsvermogen in de zoektocht naar een nieuwe plek.

Laboratorium assistentie

De materialen die beschikbaar kwamen via SOS moesten natuurlijk onderzocht en verwerkt worden. Dit gebeurde op de Erasmus MC in Rotterdam met hulp van de wildlife groep. Waar dit op zich misschien geen reden is voor een dankbetuiging, waren de sfeer, de inzet, de gezelligheid, de humor en de kunde, waarmee alle taken ter hand werden genomen, dat zeker wel. Daarom Lonneke, Lineke, Debbie en Jurre: dank lieve dames voor jullie gezelligheid en nimmer aflatende interesse in wanneer er nou eens een promotiefeestje zou komen. Marco en Peter, jullie wil ik voor dezelfde redenen bedanken maar ook zeker voor jullie assistentie bij het verwerken en analyseren van materialen en hulp bij secties, ook bij nacht en ontij en op oudejaarsdag. Het is mede aan de goede sfeer op het lab te danken dat ik er persoonlijk geen enkel probleem mee heb gehad dat het promotietraject iets langer duurde dan te doen gebruikelijk.

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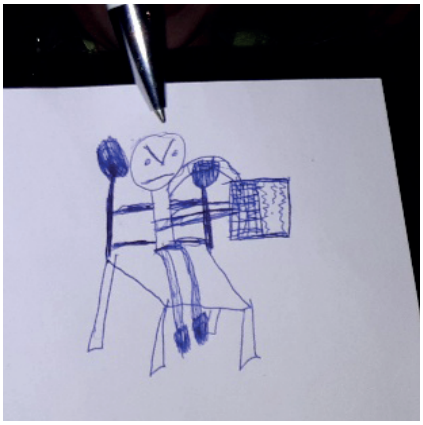
Gedoogsteun

Toen ik, vol van vreugde, mijn vrouw vertelde dat ik een promotieonderzoek mocht gaan doen viel er een verrassende stilte. Mijn vreugde werd niet gedeeld. Zwangere Maria keek me ernstig aan en zei dat ze helemaal niet blij was met het feit dat ik me voor langere tijd gedeeltelijk aan het gezinsleven ging onttrekken. De afspraak was toch dat ik een dag in de week vrij zou nemen om te helpen met het opgroeiende kroost? Ze voorzag een langdurig tijdsintensief traject. Ze had gelijk. Maria, je hebt de huishoudelijke rekening betaald voor mijn persoonlijke ambities. Ik weet het en een verschrikkelijk dank je wel alhier is een begin. Ik moet wat moois verzinnen om mijn erkentelijkheid echt inhoud te geven.

Mijn kinderen weten niet beter dat pappa met iets bezig is waarbij hij niet gestoord wenst te worden en wat hun leven lang al duurt. Nu ze al wat ouder zijn weten ze daar haarfijn de draak mee te steken. Lieve kinderen, dank jullie wel voor het mooie, puntige en geestige commentaar dat ik al die jaren van jullie heb gekregen. Ik hoop dat het jullie gegund wordt ook eens zoiets te ondernemen, als dat overeenkomt met wat jullie willen.



Supervisie

Als laatste wil ik Ab en Thijs nogmaals bedanken maar nu voor de supervisie en het gidswerk. Ab, we hebben misschien niet veel contact gehad maar het contact dat er was, was immer waardevol en plezierig. Jouw visie op wat de grote lijn is en wat de essentie is van een onderzoek, is voortreffelijk. Je aanwijzingen over de relatie tussen argumenten en conclusies en hoe je argumenten dient te formuleren waren buitengewoon behulpzaam. Ik heb het contact altijd als plezierig ervaren vanwege je scherpe energieke geest aangevuld met humor en een beetje Amsterdamse branie. Dank hiervoor.

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Als laatste

Als laatste wil ik mijn vader bedanken. Hij was het die mij interesse in en de waarde van wetenschap bijbracht. Ik herinner mij zijn promotie, dit jaar 50 jaar geleden, nog goed. Het is jammer dat hij niet meer bij mijn promotie kan zijn. Dit proefschrift draag ik op aan hem.