Phocine distemper revisited

Multidisciplinary analysis of the 2002 phocine distemper virus epidemic in the Netherlands

Opnieuw zeehondenziekte - Multidisciplinaire analyse van de zeehondenziekte epidemie in Nederland in 2002.

Proefschrift

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door

Jolianne Miriam Rijks
geboren te Kampala
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For those who patrolled the Dutch coasts to assist seals suffering from PDV-infection, and who kindly also retrieved the seals that did not make it.
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General introduction
April 1988, excess mortality of seals was observed on Anholt, a small Danish island in the Kattegat\textsuperscript{17}. This announced the start of an epidemic that killed between 18,000 and 23,000 seals in European waters in 1988, mostly harbour seals (\textit{Phoca vitulina})\textsuperscript{17,28}. The cause of this mortality was initially unknown, but through intensive research during the epidemic was shown to be a novel morbillivirus, subsequently named ‘phocine distemper virus’ (PDV)\textsuperscript{13,43,48}.

May 2002, excess mortality of seals was again observed on Anholt. This time, PDV was a known virus, and laboratory tests were available to rapidly confirm the diagnosis\textsuperscript{34}. Because PDV was immediately diagnosed, and both sero-monitoring of harbour seals\textsuperscript{34,67} and models\textsuperscript{14,23} had predicted a highly susceptible harbour seal population in 2002, decision makers were easily made aware of the likely impact the disease would have on the harbour seal population and the need to prepare for the event and monitor it.

It turned out the Netherlands would have five weeks to prepare for the epidemic: the 1\textsuperscript{st} case of PDV was diagnosed on June the 16th 2002\textsuperscript{58}. The Ministry of Agriculture, Environment and Food Quality took the coordination, consulting parties involved in seal conservation and care (e.g., Seal Research and Rehabilitation Centre SRRC, EcoMare) or wildlife health research (Department of Virology of the Erasmus MC, Dutch Wildlife Health Centre), as well as governmental coastal and island institutions (e.g., municipalities, coastguard) and others (e.g., rendering facilities) and taking the final decisions on the outbreak management and monitoring strategy.

The management and monitoring strategy set out was as follows. First, all stranded seals were to be reported to a hotline managed by the Ministry of Agriculture, Environment and Food Quality, providing a daily update on numbers of seals found stranded in different locations for outbreak monitoring. Second, live stranded seals were to be reported to seal rehabilitation centres, and these could then collect them and take them into care. Third, dead seals were to be collected by municipalities, coast guards, SRRC and Ecomare from all coasts with exception of the protected nature areas Rottumeroog-Rottumerplaat. These carcasses would subsequently be destroyed in rendering plants using high temperatures to destroy the environmental contaminants that can concentrate in seal blubber. This was mainly a precautional sanitary and a touristic comforting measure, the Wadden Sea being an important recreational area in the summertime.

Prior to seal carcass destruction in rendering plants, necropsy was carried out. In 1988, there had been no systematic necropsy of seals stranded in the Netherlands, and published data on the epidemiology, pathology and toxicology are scarce. Elsewhere, the 1988 epidemic had been better documented as summarized below.
Harbour seals and grey seals

Two seal species breed in The Netherlands: the harbour seal and the grey seal (*Halichoerus grypus*). Both haul out on sandbanks and on inter-tidal flats along tidal channels of two estuarine ecosystems, the Dutch Wadden Sea with its islands in the North, and the Delta area in the Province of Zealand in the South.8

Harbour seals reach sexual maturity around three to four years of age for females and around four to five years of age for males. They have a maximum life span of around 35 years.10,74 Births are grouped. In the Netherlands, the first pups are born at the end of May and the whelping period lasts about 37 days.54 Pups swim within hours of birth. The lactation period is three to four weeks.54 The mating period starts after pups have been weaned, and fertilization is followed by a delayed implantation of about two and a half months.10,74 Harbour seals feed on a variety of fish, including flatfish (*Pleuronectiformes*, e.g., the flounder *Platichthys flesus*), gadids (*Gadiformes*, e.g., whiting *Merlangius merlangus*), and *Clupeiformes* (e.g., herring *Clupea harengus*), and crustaceans.74,77 Maximum recorded diving depth is 508 m, and maximum recorded duration of breath-hold 31 min.58 Harbour seals moult in the summer. Usually, moult starts in yearlings, followed by sub-adults, then adult females and last adult males.10,69,74 In the summer, when harbour seals breed and moult, they appear to maintain more or less site fidelity.57,74 They haul out more frequently than in the winter, when they spend more time foraging in the North Sea.42 Numbers are therefore counted in the summer, and the proportion of seals on land is thought to represent about two-thirds of the population.56

Grey seals are sexually dimorphic. Grey seals reach sexual maturity around three to five years of age for females and around six years of age for males. Females show high fidelity to whelping sites, and give birth between September and March, depending on the site where they breed.24 In the Dutch Wadden Sea this is December-January. The lactation period is around 18 days, during which the pup can quadruple in weight. After weaning, it fasts from 10 days up to one month, loses its lanugo and develops its diving ability.24 The mating period starts after pups have been weaned. Fertilization is followed by delayed implantation of about four months.10,24 Grey seals feed on a variety of fish species, including sandeel and flatfish, and cephalopods.24 Dives greater than 300 m have been recorded and maximum recorded duration of breath-hold is 32 min.7 Grey seals haul out to moult approximately around the time of implantation, and maximum numbers are then counted (March-April in the Netherlands).

Worldwide, harbour seals are widespread, found over a latitudinal range of about 30°N to 80°N in the North Atlantic and 28°N to 62°N in the North Pacific. There are five different subspecies. The two subspecies present in the largest numbers in the North Atlantic are *P. v. vitulina* in the eastern Atlantic (approximately 100 000 in mid 1980s) and *P. v. concolor* in the Western Atlantic (40 000-100 000 in early 1990s), the boundary between them being unknown.10 The range of
the grey seal is more restricted, but not its numbers: at the turn of the 21\(^{st}\) century, there were around 130 000 in the mid and eastern Atlantic and Baltic, and over 150 000 in the western Atlantic\(^{24}\).

Along the Dutch coast, there were several thousands of harbour seals at the turn of the 20\(^{th}\) century. Their numbers subsequently plummeted to reach minimum numbers of 450 in the Dutch Wadden Sea in 1978\(^{51}\) and of 16 in the Delta area in 1981\(^{52}\). This was a result of hunting (which was only banned in the Netherlands in 1962), habitat limitation, and poor reproduction (presumably due to environmental contaminants and human disturbance)\(^{51-53}\). After that, the population started to recover and in 1987, before the 1988 epidemic, 966 harbour seals were counted in the Dutch Wadden Sea\(^{17}\).

Grey seals were historically present along the Dutch coast but were not observed there in the 20\(^{th}\) century until the 1950s. It was only in 1985 that they established a breeding colony between the Wadden Sea islands of Vlieland and Terschelling\(^{55,73}\). In 1987, before the 1988 epidemic, 71 grey seals were counted in this breeding colony\(^{71}\).

**Morbillivirus species**

Morbillivirus species belong to the *Morbillivirus* genus, *Paramyxovirinae* sub-family, and *Paramyxoviridae* family. They are enveloped, single-stranded, negative-sense RNA viruses. The genome comprises six genes, which encode eight proteins: the nucleocapsid protein (N), the phosphoprotein (P) and the two non-structural proteins C and V encoded through alternative reading frames of P, the matrix protein (M), the fusion glycoprotein (F), the haemagglutinin glycoprotein (H), and, lastly, the large protein (L). M, H, and F form the envelope of the virion. H binds to cellular receptors. H elicits a neutralizing antibody response. The H gene is one of the most variable parts of the genome. F, activated by cleavage by cellular protease, and in association with H, mediates the fusion of the viral envelope with the plasma membrane. F is essential for viral infectivity and for direct cell-to-cell spread, and the F gene is one of the most stable parts of the genome. The nucleocapsid of the virion, made of N, P, and L, bound to the RNA, is liberated by the fusion of the viral envelope with the plasma membrane. The genome is transcribed progressively by the virion-associated RNA-dependent RNA polymerase into six discrete unprocessed mRNAs by sequential interrupted synthesis from a single promoter. Full genome-length positive-sense RNA is also synthesized, and serves as a template for the replication of negative-sense genomic RNA\(^{47}\).

There are eight known morbillivirus species today: measles virus (MV), rinderpest virus (RPV), peste-des-petits-ruminants virus (PPRV), canine distemper virus (CDV), phocine distemper virus (PDV), dolphin morbillivirus (DMV), porpoise morbillivirus (PMV), and pilot whale morbillivirus (PWMV; Figure 1). The latter five are known to have infected marine mammals, and the
latter four were discovered in the last three decades. Phocine distemper virus is phylogenetically most closely related to CDV. The potential for cross-species transmission is not uncommon for morbillivirus species. During RPV or CDV epidemics involving multiple hosts species, sequence data of viral gene-fragments obtained by reverse-transcriptase polymerase chain-reaction (RT-PCR) suggested the same strain was infecting the different host species. To date, to the best of our knowledge, PDV has been isolated only from harbour seals and from mink (on a mink farm near sea in Denmark, presumably infected by contact with material from dead PDV-infected seals), though there was evidence of PDV transmission among grey seals.

### Epidemiology

During the 1988 PDV epidemic, the seals found stranded were mainly harbour seals. Grey seals rarely stranded despite their large numbers in the eastern Atlantic and Baltic. There was however evidence of seroconversion in some grey seal populations and the grey seal pup production in 1989 was lower by 6% to 24% compared to 1988. The greater susceptibility of harbour seals than grey seals to PDV infection was later confirmed experimentally.

In different areas of the North Sea, the PDV epidemic started and peaked at different times. After the island of Anholt, the infection started to occur in other areas in the Kattegat in April-May 1988; in the Skagerrak, the Danish and Dutch Wadden Sea in May; in Limfjorden, the German Wadden Sea and the Western Baltic in June; in Norway and the Southwestern Baltic, as well as in the U.K. and Ireland in August and September. In most localities, the epidemic lasted about...
two months, but in the Netherlands it was lengthier (115 days), the index case occurring on 22 May 1988, and the date of the median case being reached on 4 September\textsuperscript{17}. These temporal differences in the occurrence of the local epidemics were presumed to be due to the moment of local introduction of the infection, as dispersal rate of the disease was inversely proportional to the distance between afflicted seal herds\textsuperscript{30}.

The overall number of seals stranded along the European coasts in 1988 was around 18,000, estimated to correspond to approximately 23,000 deaths\textsuperscript{31}. In the Netherlands, 417 harbour seals were reported dead, and no grey seals\textsuperscript{17}. Estimated mortalities ranged from 10\% to 60\% among areas\textsuperscript{31}. These spatial differences in mortality were hypothesized to be due to differences in contact rates (exposure) or differences in susceptibility to fatal disease after infection\textsuperscript{23,31}.

The harbour seal strandings in 1988 showed particular sex and age patterns. In the eastern North Sea (Kattegat, Skagerrak), from June to August 1988, the proportion of males that stranded increased, and in total more males than females stranded\textsuperscript{30}. In the U.K., from July to December 1988, males also dominated in stranded seal samples\textsuperscript{26}. In the eastern North Sea, the disease was assumed to have wiped out the entire 1988-year class, and among those older than eight months to have affected the mature seals more than the immature\textsuperscript{30}. In the U.K., seals born in 1988 were underrepresented in the stranded seal sample, possibly due to lack of exposure\textsuperscript{26,29}. The sex-age patterns too were hypothesized to be due to differences either in exposure or in immunocompetence.

The source of the virus led to much speculation. Prior to the 1988 epidemic, hardly any harbour seals in the North Sea had antibodies to morbillivirus species\textsuperscript{50}. Therefore it was assumed that the infection was introduced by another species. In subsequent retrospective studies, antibodies against morbillivirus species were commonly demonstrated in samples taken before 1988 from pinnipeds in the Arctic, including harp seals (\textit{Phoca groenlandica}), and along North-west Atlantic coastline. Barents Sea harp seals had made an exceptional southward movement with incursion into the North Sea waters in the winter of 1987-1988\textsuperscript{17}. Noteworthy is the presence of neutralising antibodies against PDV in a third of the harbour seals and more than two-thirds of the grey seal sampled along the east coast of the USA in from 1980- to 1994\textsuperscript{20}.

**Pathology**

Several pathological studies were performed on the carcasses of seals that stranded during the 1988 epidemic in the U.K.\textsuperscript{2,38,46}, Sweden\textsuperscript{3,5} and Denmark\textsuperscript{32}. The seals were mostly harbour seals and rarely grey seals.

At gross necropsy, consistent findings in harbour seals were congested and consolidated lungs, and emphysema, be it pulmonary, mediastinal, pericardial, or subcutaneous, or a combination
of these. Occasional findings were oedematic or suppurative lungs, enlarged tracheo-bronchial lymphnodes, atrophy of lymphoid organs, or congestion of other organs.

Upon histology, the most consistent lesions were broncho-interstitial pneumonia, interstitial pneumonia or suppurative bronchopneumonia, and lymphocytic depletion from lymphoid tissues. Non-suppurative sometimes demyelinating encephalitis was found occasionally. Syncytial cells, intracytoplasmic and intranuclear eosinophilic inclusion bodies in various epithelial cells were present in some lesions.

Co-infections were common. These included parasites (lice, worms), bacteria (in particular *Bordetella bronchiseptica*[^3]) and several other viruses.

**Toxicology**

Environmental contaminants tend to accumulate in seal tissues because they are at the top of the food chain. The high number of seal carcasses that stranded provided an opportunity to measure levels of organohalogens in the North Sea. Published studies include data on seals stranded in 1988 in Denmark[^65], Norway[^6,63], Sweden[^8], Northern Ireland[^44] and the U.K.[^8,25,41,62], as well as from the North Sea and Baltic Sea[^75]. The compounds examined were polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT) and occasionally hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), chlordane compounds, heptachlor epoxide and heavy metals. Levels were at the high end of the range in the Baltic and southern Wadden Sea, and at the low end of the range in Norway and Northern Ireland.

Environmental contaminants, in particular polychlorinated biphenyls (PCBs), had been associated with poor reproductive performance in harbour seals[^53]. In addition, it was suspected that these contaminants may have affected the immune system, contributing to the severity and the extent of the epidemic. It was subsequently demonstrated that harbour seals chronically exposed to environmental contaminants through their diet had vitamin A and thyroid hormone deficiency[^8], as well as impaired immunological functions[^16,61]. In the ultimate stages of fasting, the levels of contaminants in liver, kidney and blood were found to increase, possibly exacerbating the effects of these contaminants[^15,75].

**Host genetics**

Harbour seal populations in the North Eastern Atlantic have been studied employing a number of methods: allozymes, multilocus fingerprinting, randomly amplified polymorphic DNAs (RAPDs), on mitochondrial control region sequencing and microsatellite DNA polymorphisms[^21,35,36,64,66]. Using the last technique, six harbour seal populations were identified: Wadden Sea; English east coast; Western Scandinavia; East Baltic; Scotland-Ireland; Iceland. The Wadden Sea popu-
lation, as well as the East Baltic population, had significantly lower average heterozygosity at microsatellite loci than the Scandinavian and Scottish populations\textsuperscript{21}. Lower genetic variation, as measured by average heterozygosity at microsatellite loci, has been associated with higher susceptibility to disease\textsuperscript{1}.

**The scope of this thesis**

Although there were several studies on the 1988 PDV epidemic, details on the epidemiological, pathological and toxicological findings in seals in the Netherlands were lacking. The aim of this thesis was to enhance our understanding of PDV infection in seals through a multidisciplinary analysis of the 2002 PDV epidemic in the Netherlands.

Chapters 2.1. to 2.3. relate to PDV epidemiology. Before the epidemic in 2001, there were around 3700 harbour seals and over 500 grey seals counted in the Netherlands\textsuperscript{70,60,71}. During the 2002 PDV epidemic, 2284 seals stranded along the Dutch coast and necropsies were performed on 1315 of them. Chapter 2.1. presents the temporal and spatial pattern of these seal strandings, and the effect of animal-related and environmental variables on the dynamics of the epidemic. Evidence for different temporal stranding patterns among stages led to a literature study on factors influencing transmission in several other morbillivirus infections (Chapter 2.2.). It also led to the creation of models with different transmission dynamics to examine whether these support stage-structured transmission (Chapter 2.3.)

Chapter 3.1. relates to PDV infection associated pathology. The large number of necropsies performed during and after the epidemic provided an unique opportunity to use a quantitative approach to the lesions caused by and associated with PDV infection.

Chapters 4.1. and 4.2. relate to environmental contaminant levels in seals that stranded during the 2002 PDV epidemic and the years before. The temporal trend of levels of different major environmental contaminants is examined in Chapter 4.1., while Chapter 4.2. specifically describes levels of polybrominated diphenyl ethers.

Chapter 5 relates to seal genetics. Samples from seals were typed for different loci, with two studies in mind. The first was whether there was a relation between the time of stranding in the PDV epidemic and homozygosity as had been observed during a dolphin morbillivirus outbreak\textsuperscript{72}; the second was whether there was a relationship between lungworm burden and homozygosity. Crude analysis of the data did not show the first, but the latter led to interesting findings.

Chapter 6 presents a discussion on the results and implications of these studies.
Reference List

General introduction


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2

Epidemiology
2.1

Epidemiology of the 2002 phocine distemper outbreak in the Netherlands

Long version

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Jolianne M. Rijks • Marco W.G. van de Bildt • Trine Jensen • Joost D.W. Philippa •
Albert D.M.E. Osterhaus • Thijs Kuiken

Department of Virology, Erasmus MC, Rotterdam, the Netherlands; Dutch Wildlife Health Centre, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands; Laboratory of Virology and Immunology, Department of Veterinary Pathobiology, Frederiksberg, Denmark.
Phocine distemper virus (PDV) infection killed more than 22,500 seals in European waters in 2002. Effects of animal-related and environmental variables on the dynamics of the epidemic in the Netherlands were determined. Along the Dutch coast, 2284 sick and dead seals—99% of which were harbour seals (*Phoca vitulina*)—were reported stranded between June and November 2002. Standardized necropsies were performed on 1315 seals. Subadults were affected earlier than juveniles and adults, and within each age category, males earlier in time than females. These differences may be explained by sex- and age-related variations in behaviour and tissue contaminant levels. Seals in Zealand were affected later than those in the Wadden Sea, probably because virus spread was slower in the smaller and dispersed Zealand population. The estimated cumulative mortality in 2002 (54%) was similar to that in 1988 (53%), suggesting that pathogenicity of PDV for harbour seals has not changed.

**Introduction**

Marine mammal morbilliviruses are among the most pathogenic infectious agents to emerge in wildlife. Phocine distemper virus (PDV) infection\textsuperscript{7,17,21} was held responsible for the deaths of about 18,000 seals in Europe in the first recorded outbreak in 1988\textsuperscript{9} and of at least 22,500 seals in the more recent outbreak in 2002\textsuperscript{26}. The high mortality caused by PDV infection in harbour seals (*Phoca vitulina*) resembles that caused by other morbillivirus infections in susceptible populations, e.g., rinderpest virus in cattle and measles virus in humans.

Epidemiological studies of the 1988 PDV epidemic\textsuperscript{12,15} showed that the level and timing of mortality of seals varied with species, sex, age, and geographical area. To explain this, haul-out behaviour was hypothesized to influence viral transmission, which was thought to be more likely on land than in water\textsuperscript{12}. However, these epidemiological studies were hampered by small sample size, and did not include the Wadden Sea. A typical characteristic of this important habitat for harbour seals is that many sites suitable for haul-out are exposed only during low tide, which influences haul-out behaviour and spatial organization.

The first objective was to determine how animal-related and environmental variables affected the dynamics of the 2002 PDV epidemic in the Netherlands, including the Dutch part of the Wadden Sea. For this, data from a large sample of stranded seals collected along the Dutch coast in 2002 were used.

Prolonged interaction between infectious agents and their host has often been associated with a reduction in pathogenicity\textsuperscript{3}. Therefore, the second objective was to compare the epidemiological characteristics, including overall mortality, of the 1988 and 2002 PDV epidemics, using both published information and our own data.
Materials and methods

Reports of stranded seals
The general public, seal rehabilitation centres, and governmental authorities reported stranded seals to a central round-the-clock telephone service. Reports included date, location (recorded as x and y coordinates of the Dutch coordinate system “Amersfoortse Rijksdriehoeksstelsel”), species (as determined by the finder), and manner of disposal.

Manner of disposal of stranded seals
Dead stranded seals were buried (a rope was tied around the body to prevent duplicate counting), incinerated, or necropsied. Seals for necropsy were identified by a uniquely numbered cattle tag and either examined directly or stored frozen for later examination. Seals for necropsy were obtained from the entire Dutch coast (Figure 1A), except for Rottumeroog and Rottumerplaat, where they were buried, and Texel and the adjacent part of mainland North Holland, where they were collected for a different study. Live stranded seals were treated at a rehabilitation centre or euthanized.

Necropsies
Species, sex, body length, and state of decomposition of each carcass were determined at necropsy. Species identification was based on examination of the teeth. Sex was based on examination of the gonads. Standard length was measured as the straight distance from tip of nose to tip of tail, with the carcass lying flat on its back. Harbour seals were divided into three age categories, based on standard length: male juveniles (age ≤ 1 yr; length ≤ 95 cm), subadults (1 yr < age ≤ 4 yr; 95 cm < length ≤ 140 cm), or adults (age > 4 yr; length > 140 cm); female juveniles (age ≤ 1 yr; length ≤ 90 cm), subadults (1 yr < age ≤ 3 yr; 90 cm < length ≤ 130 cm), or adults (age > 3 yr; length > 130 cm). State of decomposition was categorized as fresh or decomposed. Carcasses were considered fresh when abdominal organs were not or only partially discoloured due to blood imbibition and had their original shape. Carcasses were considered decomposed when abdominal organs were diffusely discoloured due to blood imbibition and were partly deformed or no longer recognizable except by their position.

Of 1315 seals necropsied in total, we selected 1096 harbour seals with a full set of data (Table 1), subsequently referred to as ‘necropsied seals’. It excluded three grey seals (Halichoerus grypus), and harbour seals with missing data. It also excluded harbour seals from Texel, Rottumeroog, and Rottumerplaat, and from the mainland coasts of North Holland and South Holland, because no or few seals from those locations were necropsied (Figure 1A).

Necropsied seals represented 56 to 73% of the stranded seals in the remaining locations (Figure 1A), and showed a similar-shaped epidemic curve to that of stranded seals (Figure 1B). Therefore, they were considered to be representative of stranded seals.
Figure 1: Spatial and temporal distribution of seal strandings in the Netherlands during the 2002 PDV epidemic. A: Spatial distribution of seal strandings. The diameter of each pie chart corresponds to the number of seals stranded at a particular location. The names of the Wadden Sea islands have been abbreviated (Tx: Texel; V: Vlieland; Ts: Terschelling; A: Ameland; S: Schiermonnikoog; R: Rottumeroog en Rottumerplaat) B: Weekly stranding rate of all recorded seals compared to that of seals necropsied.
Table 1: Age and sex distribution of harbour seals stranded during the 2002 PDV epidemic in the Netherlands.

<table>
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<td>281</td>
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<td>172</td>
<td>(0)†</td>
<td>- -</td>
<td>- -</td>
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</tr>
<tr>
<td>Friesland mainland</td>
<td></td>
<td>93</td>
<td>217</td>
<td>159</td>
<td>8 21</td>
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<td>27 38</td>
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<td>194</td>
<td>138</td>
<td>18 34</td>
<td>28 50</td>
<td>24 40</td>
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<tr>
<td>North Holland</td>
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<td>124</td>
<td>97</td>
<td>(1)†</td>
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<tr>
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<td>18</td>
<td>(8)†</td>
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<td></td>
<td>706</td>
<td>2284</td>
<td>1096</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Kilometers of coastline, derived from Camphuysen 1997
†Age category and sex for stranded seals with missing observations were imputed using those of seals that stranded in the same location and the same or closest weekly date.
‡Not included in the analysis of necropsied seals.

Wind and spring tide

The daily wind factor was calculated by multiplying average daily wind force (m/s) at Den Helder, North Holland (obtained from the Royal Netherlands Meteorological Institute) with its coefficient. Coefficients depended on the average angle of the wind to the WSW-ENE line, and ranged from 0, when parallel to the line, to 4, when at right angles to it. Coefficients were considered positive for winds north of the line and negative for winds south of the line. To analyze the effect of spring tide, the number of strandings on the day of spring tide and the two subsequent days were compared to the number of strandings on other days.

Statistical analysis

Effect of age category and location (overall and per age category) on temporal distribution of strandings was examined with the Kruskal-Wallis (K-W) test with multiple comparisons. Effect of sex within each age category and of spring tide on temporal distribution of strandings was examined with the Mann-Whitney U test. To test whether the proportion of decomposed carcasses increased with time, we used the chi-square test for linear trends. Effect of age category, sex, and state of decomposition on spatial distribution of strandings was examined with Pearson’s chi-square test. Age category, sex, and state of decomposition for stranded seals with missing observations were imputed using those of necropsied seals that stranded in the same location and on the same or closest weekly date. P-values ≤ 0.05 were considered to be statistically significant (SPSS for Windows, SPSS Inc., Chicago, Ill., USA).
Results

**Overall**
The Dutch index case was found on Vlieland on 16 June 2002. The stranding rate increased rapidly from the last week of July, peaked at the fourth week of August (370 stranded seals), subsided and peaked again at the third week of September (301 stranded seals), returned to low values by the second week of October, and back to pre-epidemic levels during November 2002. Central reporting was discontinued on 29 November 2002. (Figure 1B, Table 2).

During the epidemic, 2284 seals (2154 dead, 130 live) were reported stranded along the Dutch coast. Most (2279 of 2284) were identified as harbour seals, and the remaining 5 as grey seals, 3 of which were necropsied. The cumulative mortality was calculated as 54% (Table 2). Of the stranded seals, 2166 of 2284 (95%) were found on the islands or mainland bordering the Wadden Sea (Figure 1A).

**Table 2**: Comparison of the overall characteristics of the 1988 and 2002 PDV epidemics in the Netherlands.

<table>
<thead>
<tr>
<th>Variable</th>
<th>1988</th>
<th>2002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date index case</td>
<td>22 May</td>
<td>16 Jun</td>
</tr>
<tr>
<td>Date median case</td>
<td>4 Sep</td>
<td>2 Sep</td>
</tr>
<tr>
<td>Central epoch (days)</td>
<td>115</td>
<td>93</td>
</tr>
<tr>
<td>No. found stranded</td>
<td>417</td>
<td>2284</td>
</tr>
<tr>
<td>No. counted in pre-epidemic year</td>
<td>966</td>
<td>3595</td>
</tr>
<tr>
<td>No. counted in post-epidemic year</td>
<td>535</td>
<td>2365</td>
</tr>
<tr>
<td>Average annual population growth in pre-epidemic years (%)</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>Estimated cumulative mortality (%)</td>
<td>53</td>
<td>54</td>
</tr>
</tbody>
</table>

*Dietz et al, 1989⁹
†Extrapolated from data in Reijnders & Brasseur, 2001¹²
‡Data given in Wadden Sea Newsletters¹⁴,¹⁶
§ Reijnders et al, 1997²⁵
¶ Ries et al, 1999³⁰
#Calculated as follows: ((number of live seals counted in pre-epidemic year + average annual growth in pre-epidemic years) – (number of live seals counted in post-epidemic year – average annual growth in pre-epidemic years)) / (number of live seals counted in pre-epidemic year + average annual population growth in pre-epidemic years).

**Age category and sex**
Age category and sex affected the temporal distribution of strandings. The median stranding date varied significantly among age categories ($X^2 = 44.36$, $df = 2$, $P \leq 0.001$). The median stranding date for subadults was significantly earlier than that for juveniles and adults (K-W multiple comparisons between age classes, $P \leq 0.05$; Figure 2A); however, the median stranding dates for juveniles and adults did not differ significantly. Within each age category, the median stranding date for males was significantly earlier than that for females in juveniles ($Z = 4.36$, $P \leq 0.001$), subadults ($Z = 3.91$, $P \leq 0.001$), and adults ($Z = 5.48$, $P \leq 0.001$) (Figures 2B to 2D).
Age and, to a lesser extent, sex also affected the geographical distribution of strandings. The proportion of seals of each age category that stranded varied significantly among Wadden Sea locations ($X^2 = 96.34, df = 10, P \leq 0.001$). The highest proportion of juveniles and adults stranded at mainland Groningen; the highest proportion of subadults stranded on Vlieland (Figure 3A). Looking at each age category separately, the number of seals stranded per km of coastline varied significantly among locations for juveniles ($X^2 = 114.56, df = 5, P \leq 0.001$), subadults...
(X^2 = 396.99, df = 5, P ≤ 0.001), and adults (X^2 = 141.97, df = 5, P ≤ 0.001). For all age categories, the greatest positive discrepancy between observed and expected values was found for Schiermonnikoog, where 2.2 to 3.1 (depending on age category) more seals stranded per km than would be expected had the seals been evenly distributed per km coast (Figures 3B to 3D). Within each age category, the proportion of males to females varied significantly among locations only for adults (X^2 = 20.45, df = 5, P ≤ 0.001). Ameland had the highest proportion of adult males, and Vlieland the lowest (Figure 3D).

**Figure 3**: Effect of age category and sex on spatial distribution of stranded harbour seals. The diameter of each pie chart corresponds to the number of harbour seals stranded per km coastline at a particular location. A: Stranded harbour seals, by age category. B: Juveniles, by sex. C: Subadults, by sex. D: Adults, by sex.

**Location**

Location affected the temporal distribution of strandings, overall and within age categories. Overall, the median stranding date varied significantly among locations (X^2 = 85.46, df = 6, P ≤ 0.001). The median stranding week at Zealand (week 39) was significantly later than that for all Wadden Sea locations. Within the Wadden Sea area, the median weekly stranding date
for Ameland (week 35) was significantly earlier than that for all other locations (weeks 36-37) except for Terschelling (also week 35, but with different temporal distribution of strandings) (K-W multiple comparisons between locations, $P \leq 0.05$; Figure 4). Within each age category, the weekly median stranding dates varied significantly among locations for juveniles ($X^2 = 13.77$, df = 6, $P \leq 0.05$), subadults ($X^2 = 106.07$, df = 6, $P \leq 0.001$) and adults ($X^2 = 14.98$, df = 6, $P \leq 0.05$). The most significant differences were seen in the subadult age category, where the trends were similar to those observed above for stranded seals overall (K-W multiple comparisons of subadults between locations, $P \leq 0.05$).

**Wind**

Wind acted as a confounding factor on the stranding rate of seals. Periods of southerly wind corresponded with decreased overall stranding rates (Figure 5), and vice versa for periods of northerly wind. This is shown by the trough in between the two major modes of the epidemic curve (Figures 1B, 5) which corresponds to a 9-day-long period of continuous southerly wind (Figure 5).
**Spring tide**

Spring tide did not act noticeably as a confounding factor on stranding rates. The stranding rate on the day of spring tide and the two following days was not significantly higher than that on all other days ($Z = 0.09, P > 0.05$).

**State of decomposition**

State of decomposition as a measure of length of time between death of a seal and its detection had a confounding effect on stranding rates, both temporally and spatially. From July to October, the overall proportion of decomposed seals differed significantly among months ($X^2 = 28.04, df = 3, P \leq 0.001$) and increased significantly with time ($X^2 = 23.78, df = 1, P \leq 0.001$) (Figure 6A).

Eighty-seven percent of the carcasses that stranded in the last week of October (Figure 1B) were decomposed. This small peak of strandings followed the most severe storm in the Netherlands since 12 years, with prevailing WSW wind averaging 15.7 m/s (www.knmi.nl).

The proportion of decomposed carcasses varied significantly by location ($X^2 = 225.57, df = 6, P \leq 0.001$), with high proportions of decomposed carcasses on the mainland coasts of Friesland and Groningen and on Schiermonnikoog, and low proportions on the remaining Wadden Sea islands (Figure 6B).

**Figure 6:** Temporal and spatial variation in state of decomposition of stranded harbour seals. A: Proportion of decomposed seals per month, overall and per location. B: Proportion of decomposed seals per location. The diameter of each pie chart corresponds to the number of seals stranded per km coastline at a particular location.
Discussion

Several animal-related and environmental variables affected the stranding pattern of seals during the 2002 PDV epidemic in the Netherlands. Animal-related variables affecting the stranding pattern were species, age, and sex. First, the main species that stranded was the harbour seal. Grey seals were likely exposed to PDV because their numbers have increased recently in the Netherlands and they haul out together with harbour seals. Despite this, grey seals rarely stranded, confirming experimental findings that PDV infection is more pathogenic for harbour than for grey seals.

Second, age significantly affected the pattern of strandings, both temporally and spatially. The main effect of age on temporal pattern of strandings was that the median stranding date of juveniles and—especially female—adults was later than that of subadults (Figure 2A). Possible explanations include differences in behaviour and contaminant burdens.

Behaviour of females with pups may have played a role because the timing of pupping—which peaks on 1 July in the Dutch Wadden Sea—and the subsequent 3- to 4-week-long lactation period corresponded to the beginning of the epidemic (Figure 1B). At haul-out sites, lactating females and their pups either form distant groups away from the main body of the seal colony, or mix with other seals but keep greater distance from other seals. Females with pups also are more sedentary than males or females without pups, and reduce their range size from a few days before parturition until 2.5 to 3.5 weeks after parturition. Females with pups have fewer new contacts with other seals than males and females without pups, and limit their social play to their pups. Following weaning, pups do not interact with each other. In contrast, subadults (“yearlings”, “juveniles” and “subadults” in Renouf and Lawson’s categorization) display considerably more social play than other age categories. Together, these behavioural differences would have decreased the contact rates of adult females and their pups with other seals and lowered their risk of contacting infected seals at the beginning of the epidemic, resulting in later median stranding dates compared to adult males and subadults (Figures 2B to 2D). This corresponds to the study of Härkönen and Harding, who found that adult females died later in the 1988 epidemic in Sweden than subadult females and adult males because adult females were less mobile at the start of the epidemic.

We question the assumption made after the 1988 epidemic that viral transmission is more likely to occur on land than in the water. Based on behaviour studies, seals have closer contact—and therefore more likely transmission of virus—in the water than when they are hauled-out on land, where there is a very low rate of social interaction. Close contact in the water may occur during social play in the period before hauling out, during fights between males, and during mating between adult males and adult females. Furthermore, social interaction in the water, which often involves body contact and often is muzzle to muzzle, is more likely to allow viral
transmission than the social interaction on the land, which usually involves agonistic behaviour such as fore flipper waving and head thrusts rather than body contact\textsuperscript{10}. In order to improve our understanding of age- and sex-related differences in behaviour on PDV transmission among seals, behaviour studies are required that measure the degree and type of contact required for viral transmission, and the rates of such contacts among seals.

In addition to differences in behaviour, differences in contaminant burdens also may have played a role in the later median stranding date of adult females and juveniles. The contaminant levels in the tissues of seals that died in the 1988 PDV epidemic were considered sufficiently high to cause immunosuppression and thus increase their susceptibility to PDV\textsuperscript{8,32}. Because adult females lose part of their contaminants through pupping and lactation, their contaminant levels are lower overall than those in adult males\textsuperscript{1}. Levels in juveniles are generally lower than those in older animals, because there has been no accumulation with age\textsuperscript{1}. Therefore, adult females and juveniles may have been less immunosuppressed than adult males and subadults and may have succumbed more slowly after PDV infection. Since 1988, pollutant levels in seal tissues from the Netherlands have not been monitored. This would be required to determine whether levels have changed, and to investigate sex- and age-related variation.

There was a significant effect of age on spatial distribution of strandings. High proportions of juvenile and adult seals stranded in Groningen; Groningen includes the coastline of the Eemsmond, a core breeding area\textsuperscript{29}. High proportions of subadults stranded in the western part of the Dutch Wadden Sea; this area is assumed to have an influx of migrating pups\textsuperscript{30} (Figure 3A). For all age categories, the density of harbour seal strandings (numbers per km coastline) were the highest on Schiermonnikoog (Figures 3B to 3D); this coincides with the summer distribution of harbour seals in the Dutch Wadden Sea, which is highly skewed towards the eastern part of the Wadden Sea\textsuperscript{16,30}.

The third animal-related variable, sex, had a significant effect on the temporal pattern of strandings. Within each age category, males consistently stranded earlier than females (Figures 2B to 2D). These findings correspond with those for the 1988 epidemic in Denmark, where more males than females died during the first half of the epidemic, and more females than males in the second half\textsuperscript{15}. A possible explanation is different behaviour between males and females. In individually identified seals, the turnover rate at haul-out areas is significantly higher for males than for females\textsuperscript{10}. Adult males travel widely during the pupping period\textsuperscript{39} and have abundant male-to-male body contact in the water\textsuperscript{33}, while adult females with pups are separated from the rest of the herd with little interaction and a small range (see references above). Subadult males have the longest and most aggressive interactions with each other\textsuperscript{34}. Together, these sex-related differences in behaviour imply a higher contact rate for males than for females, increasing the risk of infection.
Besides the animal-related variables discussed above, the environmental variables location, wind, and state of decomposition also affected the stranding pattern during the 2002 PDV epidemic. First, location had a significant effect on the temporal pattern and overall number of strandings. Most importantly, the median stranding date in Zealand was nearly one month later than those in locations in the Wadden Sea area (Figure 4). This is probably because seals in Zealand are fewer and more widely dispersed than in the Wadden area⁴, so that there is a lower chance of virus spread.

Second, wind had a strong effect on stranding rate (Figure 5), probably because dead seals were in the top water layer, which shows parallel drift to surface winds. A similar effect of wind on strandings has been shown for seabirds⁶, and was also suspected—but not demonstrated—for seals in Denmark and Sweden during the 1988 PDV epidemic¹⁵. These findings show that wind can have a strong confounding effect on stranding rate, and needs to be considered when interpreting stranding patterns of carcasses on the shores of large water bodies.

Third, the proportion of decomposed seal carcasses varied both temporally and spatially. Temporally, the proportion of decomposed seal carcasses increased significantly during the course of the epidemic (Figure 6A). This is probably because recovery of seal carcasses was not 100% (some seals may have died at sea), so that, as the epidemic progressed, a higher proportion of stranded carcasses consisted of seals that had died before the previous shore survey. A similar trend was visible in the U.K. during the 1988 PDV epidemic¹². These findings suggest that stranding rate became a less accurate estimate of mortality rate as the epidemic progressed, and thus the right-hand limb of the epidemic curve (Figures 1B and 2A-2D) needs to be interpreted with caution.

Spatially, the proportion of decomposed seal carcasses was highest on the mainland coasts of Friesland and Groningen and on Schiermonnikoog (Figure 6B). The proportion of decomposed carcasses may have been higher at the mainland coasts because they are lined by extensive mudflats, so that carcasses were more difficult to detect than on the sandy beaches of the Wadden Sea islands. The proportion of decomposed carcasses may have been higher on Schiermonnikoog because it received carcasses that floated away from the adjacent islands Rottumeroog and Rottumerplaat, where carcasses were not removed.

Besides determining the effects of animal-related and environmental variables on the stranding pattern of the 2002 PDV epidemic, the overall epidemiological characteristics of the 2002 PDV epidemic in the Netherlands to that in 1988 were also compared. The timing of the epidemic and the cumulative mortality were similar in both years (Table 2). The exception is the index case, which was detected about one month later in 2002 than in 1988. The similarity between estimated cumulative mortality in 1988 (53%) and 2002 (54%) suggests that the pathogenicity of PDV for the harbour seal population has not changed noticeably. Perhaps a single exposure
to PDV, as occurred in 1988, compared to continual exposure to a range of other pathogens, e.g., lungworms, is insufficient to cause changes in the inherent resistance of the population. A more detailed examination of the genetic composition of both the virus and the harbour seal is needed to exclude changes in the host-pathogen relationship.

**Acknowledgements**

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Epidemiology of the 2002 phocine distemper outbreak in the Netherlands


Factors affecting morbillivirus transmission

Jolianne M. Rijks • Albert D.M.E. Osterhaus • Thijs Kuiken

Department of Virology, Erasmus MC, Rotterdam, the Netherlands; Dutch Wildlife Health Center, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.
Introduction

In the last two decades, four diseases emerged in marine mammals, each caused by novel viruses belonging to the morbillivirus genus. High mortality was often seen in the host populations in which these diseases emerged.

The emergence of these novel diseases raises questions about the maintenance and transmission of these morbillivirus infections in and between host populations, and leads to concern about the impact of these diseases on host populations. Such issues are being dealt by using molecular techniques to explore the origin and pathogenesis of morbillivirus species and strains, and by developing models to understand multiple-host epidemiology and to assess effects on host populations.

Nevertheless, the understanding of morbillivirus transmission among marine mammals is still far from complete. In order to get a better understanding of which factors are involved in morbillivirus transmission among marine mammals, we first reviewed eight factors affecting more known morbillivirus species in terrestrial mammals. We subsequently applied this knowledge to morbillivirus species in marine mammals, and particularly to phocine distemper virus (PDV) in harbour seals (Phoca vitulina). The eight factors we studied were the susceptibility, the infectiousness and the behaviour of the hosts. They were also the infectivity and virulence of the virus and its stability outside the host. Finally, they were the routes of excretion and infection. These factors are linked to aspects of the pathogenesis, such as tissue tropism, and these are dealt with accordingly.

**Figure 1:** Factors within hosts and during transmission determining the potential for infection by morbilliviruses. **Routes of infection** are the sites (portals of entry) and modes (direct or indirect contact) of infection. **Infectivity of the virus** its ability to establish infection. **Susceptibility of the host** refers to its predisposition to become infected. **Virulence of the virus** is the degree to which the virus is invasive and/or damaging to the host. **Infectiousness of the host** refers to the duration of the period that the host is infective, and to the amount of virus that it can transmit to another host. **Routes of excretion** are the sites (portals of exit) by which the virus leaves the infected host. **Stability of the virus** is the length of time for which it can remain infective outside the host. **Behaviour of hosts** influences the type and intensity of exposure (route, duration, dose), and may change as a result of infection.
The data were obtained and collated from publications describing natural or experimental infections in the best-documented morbillivirus-host pairs, i.e., measles virus infections in man (MV-human) and monkeys (MV-monkey); rinderpest virus infections, mostly in cattle (RPV-cattle); and canine distemper virus infections, mostly in dogs (CDV-dog) and ferrets (CDV-ferret).

**Routes of infection, and first infected cells**

The route of infection of the virus into a susceptible host has two aspects: the site of infection (portal of entry) and the mode of infection, i.e., direct or indirect contact. Direct contact includes infections by direct physical contact between hosts, by spread of droplets between hosts, and by transplacental transmission. Indirect contact includes infections by aerosols, vectors, fomites and infected carcasses, i.e., exposures that do not require physical nearby presence of a live infectious host.

The reported efficiency of routes of infection in different virus-host pairs is summarized in Table 1 (MV-human\textsuperscript{19,25,105,106}; MV-monkey\textsuperscript{144}; RPV-cattle\textsuperscript{33,36,73,75,150}; RPV-pigs\textsuperscript{129}; CDV-dogs\textsuperscript{4,46,50,84}; CDV-ferret\textsuperscript{45,58,89}; PDV-harbour seals\textsuperscript{65}). A distinction is made between routes identified under natural and experimental conditions. Natural morbillivirus infection usually involved close proximity exposure in which several routes could be active simultaneously. Only two routes could be singled out, direct transplacental (confirmed route) and indirect aerosol, presumably to the respiratory tract (highly probable route). The latter nearly always involved closed or poorly ventilated spaces (CDV-ferret\textsuperscript{45,58}), or air currents and superspreaders (CDV-dog\textsuperscript{46}), or both (MV-measles\textsuperscript{19,48,107}). Experimental studies were more successful in their efforts to assess the efficiency of single sites using artificial modes. The disadvantage is that they do not necessarily reflect natural situations of direct or indirect contact. For example, in some experimental studies animals were infected by injection of infectious fluid, which mimics infection via bites or vectors. However, these routes of infection have not been reported in real life for morbillivirus species.

Another indication for the route of infection can be the first infected cells. The very first cells to be infected are sometimes alleged to be respiratory tract epithelial cells, but have not been precisely identified to date for any of the morbillivirus species. Three early pathogenesis studies sacrificed animals to identify organs and cells containing virus before measurable viremia, the routes of infection being natural by contact with infected animals or experimental by infectious aerosol or intranasal inoculation (RPV-cattle\textsuperscript{110,140}; CDV-dogs\textsuperscript{4}; CDV-ferrets\textsuperscript{89}). Virus or viral antigen was detected in regional lymph nodes of the head and of the lower respiratory tract (Figure 2). In two of these studies, the infected cells in the lymph nodes were morphologically identified as mononuclear cells\textsuperscript{4} or reticular cells\textsuperscript{89}. Two recent studies on MV suggested a new hypothesis for the initial spread of MV which is that CD150+ dendritic cells with DC-SIGN receptors could be
Table 1: Documented single routes of infection: “+” = an effective route of infection, generally requiring small quantities of infectious material; “±” = a possible but rare route of infection, generally requiring large quantities of infectious material; “-” = an unsuccessful route of infection in the documented experiments.

<table>
<thead>
<tr>
<th>Route of infection</th>
<th>Virus host-pairs for which the route of infection is documented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MV-</td>
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<tr>
<td>Mode of infection</td>
<td>human</td>
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<tr>
<td>Natural circumstances:</td>
<td></td>
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<tr>
<td>Direct</td>
<td>Transplacental</td>
</tr>
<tr>
<td>Indirect</td>
<td>Aerosol*</td>
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<tr>
<td>Experimental set-ups:</td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td>Transplacental</td>
</tr>
<tr>
<td>Direct (drops of infectious fluid or swab with infectious material)</td>
<td>Nasal mucosa</td>
</tr>
<tr>
<td>Direct (drops of infectious fluid)</td>
<td>Conjunctiva</td>
</tr>
<tr>
<td>Direct (variety of inoculates)</td>
<td>Digestive tract</td>
</tr>
<tr>
<td>Direct (variety of inoculates)</td>
<td>Vagina</td>
</tr>
<tr>
<td>Direct (application of infectious fluid)</td>
<td>Open wound</td>
</tr>
<tr>
<td>Direct (injection)</td>
<td>Intact skin</td>
</tr>
<tr>
<td>Indirect</td>
<td>Aerosol*</td>
</tr>
<tr>
<td>Indirect</td>
<td>Biting insect‡</td>
</tr>
</tbody>
</table>

* = presumably to the respiratory tract.
‡ = presumably intradermal.
Factors affecting morbillivirus transmission

responsible for early transport of MV from the site of infection to lymphoid organs. One of these identified DCs as major target cells for MV using a recombinant MV strain expressing enhanced green fluorescent protein (EGFP); the other identified DC-SIGN as a new attachment receptor for MV that could enhance viral transmission to CD150+ lymphocytes. If confirmed, DCs are a possible candidate for first infected cells.

The route of infection sometimes affected the incubation period, but did not seem to affect disease severity or the proportion of deaths (CDV-dogs; CDV-ferrets). Incubation periods in individuals infected by exposure to an infectious host (site-mode and dose unknown) were generally longer and more irregular than those in individuals inoculated by a successful route using a high dose of virus (MV-monkey; RPV-cattle; CDV-ferrets). This may be less an effect of route than of dose (cf. infectivity).

Figure 2: Schematic representation of the stages of spread of virulent morbillivirus strains in host tissues in relation to stages of disease and the time of appearance of neutralizing antibodies, derived from three pathogenesis studies (CDV-ferrets; CDV-dogs; RPV-cattle). The squares (and numbers) in the horizontal bars indicate the days after inoculation. The stages of disease are the incubation period (light grey squares), the clinical period (dark grey squares; slanted hatched squares when clinical signs can become less perceptible) with onset of fever (black triangles), and convalescence period (period in which the virus is cleared from tissues, white squares). The three morbillivirus host-pairs show a consistent sequence in the spread of virus to different tissues, namely from regional lymph nodes of the head and of the lower respiratory tract (1st stage of spread), via low-grade viremia to other lymphoid tissues (2nd stage of spread), and then via high-grade viremia becoming widespread in numerous organs in the body (3rd stage of spread). In ferrets, very susceptible to CDV infection, death generally occurred before neutralizing antibodies (Nabs) appeared. In dogs, NAbs appeared before the virus reached high titers in multiple organs, creating two distinct categories of dogs: those with sufficient immune response clear the infection, the others ultimately succumb between 3 weeks and 2 months p.i. In cattle, NAbs appeared well after the virus reached high titers in multiple organs, and within a few days cattle either died or recovered (cleared the infection). Cattle with sufficient immune response clear the infection sometimes succumbed to the severity of their lesions.
Infectivity, and tropism of the virus

The infectivity of the virus is its ability to establish infection, measured by the number of virus particles required to instigate infection (high infectivity when few virus particles are required). The tropism of the virus describes its affinity for certain cells or tissues, and is linked to the presence of specific cellular receptors. A pre-requisite for instigating infection is exposure of the host to an infective dose.

In inoculation experiments, infectivity was high, e.g., 1 TCID$_{50}$ of MV inoculated intra-tracheally was sufficient to infect a cynomolgus macaque (Macaca fascicularis) with MV$^{44}$. Infectivity by contact exposure could not be measured, but early studies indicated humans generally required fairly close (same living quarters) and lengthy (hours to days) exposure to MV cases$^{80}$ as did cattle in experimental RPV contact infections$^{28,140}$. Infectivity of CDV by contact exposure in dogs and ferrets may be higher$^{45,46}$.

Given that infectivity could be high, the effect of size of the infecting dose on infection and disease parameters in susceptible hosts was reviewed. Virulent strains dosed within the lower limits of infection caused either disease or no infection, but not sub-clinical infection (RPV-cattle$^{36}$; CDV-ferret$^{9,85}$). Virulent strains in low but infective doses led to longer latent and incubation periods than high doses, but did not reduce the peak level of viremia (MV-monkey$^{144}$), and did not seem to cause a difference in disease severity or mortality (RPV-cattle$^{87}$). However, mild RPV-strain in low doses did produce lower levels of PBMC-associated viremia in cattle than in high doses$^{69}$.

These experiments used single infecting doses rather than repeated doses. The higher death risk of within household secondary MV cases compared to the primary cases$^{1}$ suggests that the effects of repeated exposure during one infection need further examination.

With regard to tropism, two recent studies with recombinant morbillivirus strains expressing EGFP stressed the crucial role of replication in lymphocytes (MV-monkey$^{46}$; CDV-ferret$^{148}$), the importance of which was not fully recognized from earlier work. Re-examination of earlier experimental pathogenesis studies on host-morbillivirus pairs nevertheless showed a consistent pattern of virus infection and replication in cells of primary and secondary lymphoid organs, including mucosa-associated lymphoid tissue, before other cells of organs such as epithelial cells of the gastro-intestinal, respiratory or uro-genital tract (MV-monkeys$^{131}$; RPV-cattle$^{110}$; CDV-dogs$^{4}$) (Figure 2).

This predilection of wild-type morbillivirus species for cells of lymphoid organs is in agreement with the high affinity of these viruses for the cellular receptor SLAM (signalling lymphocyte
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activation molecule, also known as CD150 in V. Various cells of the human and monkey immune system have SLAM receptors, as do peripheral blood mononuclear cells (PBMC) of cattle and of dogs (MV+RPV+CDV in V. MV-monkey in V; RPV-cattle in V). In contrast, epithelial, endothelial and neuronal cells do not have SLAM (MV-human and the receptors involved in infection of such cells have not been fully identified. There is evidence in support of the existence of other receptors for which the virus has less affinity (MV-human). Furthermore, high affinity of virus for receptors may not be crucial for spread of virus to adjacent cells. Indeed, infected epithelial cells were often grouped in foci in the skin (CDV-ferrets in V) and in the gastro-intestinal mucosa (RPV-cattle in V.); also, epithelial infection was often confluent with sites of sub-epithelial reticulo-endothelial or lymphocytic infection (MV-human; MV-monkey; RPV-cattle).

Sequence results of gene fragments by RT-PCR suggested that during an epidemic multiple host species were infected by the same strain of RPV or CDV. Relevant to cross-species transmission could be the observation that MV, CDV and RPV grew most efficiently in tissue culture cells expressing the SLAM of their usual host (e.g., MV grew more efficiently on cells expressing human SLAM than on cells expressing dog SLAM or cattle SLAM), but could also use SLAM of non-host species as receptors (e.g., MV or RPV and Dog SLAM). A single amino acid substitution on the H-protein was enough to allow a CDV-dog strain isolated using dog SLAM to interact with marmoset SLAM.

Host susceptibility

The susceptibility of a host is a property of the host and refers to its predisposition to become infected. The predispositions to infection and to severe disease and mortality are often compounded in the term susceptibility in morbillivirus literature. Susceptibility varies between host species and between individuals, and it is modified after vaccination or the first infection.

Generally, several host species have been shown to be susceptible to infection by a given morbillivirus species under non-experimental conditions (Table 2; MV; RPV; CDV; CDV; CDV; CDV). Sequencing of gene fragments by RT-PCR and phylogenetic analyses have demonstrated the transmission of strains of morbillivirus species between host species, and geographical clustering of strains rather than clustering per host species (RPV; CDV; CDV). Inversely, some host species have been shown to be susceptible to multiple morbilliviruses, e.g., sheep and goats to RPV and to PPRV. These two features make the origin of the emerging virus species uncertain, and point to the potential risk of a host species being infected by a novel morbillivirus species after eradication of the current one.
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Table 2: Host species susceptible to infection by the measles virus, rinderpest virus or canine distemper virus under non-experimental conditions

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measles virus</td>
<td>• Humans are susceptible to natural MV infection.61</td>
</tr>
<tr>
<td></td>
<td>• Non-human primates are not infected with measles in the wild but are</td>
</tr>
<tr>
<td></td>
<td>susceptible to natural infection following their capture by humans127,157.</td>
</tr>
<tr>
<td>Rinderpest virus</td>
<td>• Cattle and water-buffalo (Bovinae) are highly susceptible to natural RPV</td>
</tr>
<tr>
<td></td>
<td>infection121. Some cattle breeds and populations may be more susceptible to</td>
</tr>
<tr>
<td></td>
<td>RPV infection than others 123.</td>
</tr>
<tr>
<td></td>
<td>• Numerous other artiodactyl species are susceptible to natural RPV</td>
</tr>
<tr>
<td></td>
<td>infection, albeit to different degrees2,111,112.</td>
</tr>
<tr>
<td>Canine distemper virus</td>
<td>• Many carnivore species are susceptible to infection by CDV153. Whether</td>
</tr>
<tr>
<td></td>
<td>within a given species some breeds are more susceptible to CDV than others</td>
</tr>
<tr>
<td></td>
<td>is subject to discussion49,57,96,104,108.</td>
</tr>
<tr>
<td></td>
<td>• Natural CDV outbreaks have also been reported in non-carnivores,</td>
</tr>
<tr>
<td></td>
<td>namely in primates (Japanese monkey Macaca fuscata159) and artiodactyls</td>
</tr>
<tr>
<td></td>
<td>(collared peccaries – Tayassu tajacu).</td>
</tr>
</tbody>
</table>

Though multiple hosts may be susceptible to infection, it is thought that only one or two host species are the reservoir of a given morbillivirus species, e.g., humans for MV61; cattle and domestic water-buffalo for RPV121. This concept is corroborated in particular by the success of the on-going rinderpest eradication campaign which had focused on vaccination of cattle and domestic buffalo121. These ‘reservoir’ host species are all highly susceptible to both infection and disease.

Host species susceptibility to infection, severe disease and mortality typically clustered at the sub-family level for RPV infection in Artiodactyla (Figure 3, derived from Plowright (1982)112 and Anderson (1992)2). Individual host susceptibility to severe morbillivirus-associated disease and mortality was particularly enhanced in three situations. First, when another pathogen was concurrently present, e.g., Babesia spp. (RPV-cattle109). Second, when certain immune deficiencies pre-existed, e.g., acute lymphoblastic leukemia (MV-human11), or when for some reason the immune response to morbillivirus infection was only partially sufficient. For example, CDV-infected dogs in which titers of neutralizing antibodies developed with delay evolved into neurological cases within two months of infection4, and MV-infected humans who did not exhibit rash often had severe giant cell pneumonia44. Third, when vitamin A was deficient during disease, providing a vitamin A supplement reduced disease severity and mortality in two virus-host pairs, humans with MV11 and ferrets with CDV120.

Susceptibility is modified after vaccination or the first infection. Re-infection can occur and boost neutralizing antibody titres, but will usually be sub-clinical. This is due to cross-protection between strains, which to date is not considered to be significantly affected by the immunological strain differences detected serologically (MV-measles100, RPV-cattle109,
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CDV-dogs 53). Serological tests have shown that there is a threshold level of acquired immunity below which MV re-infection is likely to occur in humans, with no apparent relation between reaching this threshold and the period since immunisation or natural infection100. In an enzootic situation, there may also be a slot in time in which infection in some young animals with waning maternal antibodies could develop as sub-clinical or mild rather than severe disease (RPV-cattle113).

Virulence of the virus

The virulence of the virus is a property of the virus and is the degree to which the virus is invasive and/or damaging to the host. It relates to the severity of disease and the risk of death in the infected host.

Different strains of virus exist within each morbillivirus species and these can differ in virulence (MV61; RPV124,96,112; CDV5). Attenuation of virulence of strains of morbillivirus species has been achieved in the laboratory by multiple passages in tissue culture, eggs or animals of a different host species than the strain was isolated from. Ability of the virus to spread by contact may be lost during these procedures. Attenuation may apply only to the host of origin, and may be reversible(MV61; RPV121,139; CDV5,57,62).

In addition, mild wild-type strains have been documented for RPV in East Africa21,92,109,125,138,141.

Figure 3: East African Artiodactyla grouped at sub-family level for their susceptibility to rinderpest virus infection and disease (“n = …” indicates how many host species of the sub-family were discussed with regards to their susceptibility to rinderpest virus112,2).
Cattle infected with these mild wild-type strains experienced less severe or even no clinical disease and there was little to no mortality\textsuperscript{5,109}. Two hypotheses for the emergence of mild wild-type RPV strains in the field have been put forward in the literature. The first hypothesis bears similarity to the process of attenuation in the laboratory, advancing that the virulence of strains for cattle is modified by passage of RPV in non-cattle host species, e.g., wildlife. In multi-species RPV epidemics involving wildlife, the patterns of spread of the disease in wildlife suggested transmission occurred mostly among hosts of the same species\textsuperscript{82,95}, allowing a within-species passage history before cross-species transmission events. Strains isolated from wildlife either stayed consistently mild\textsuperscript{118} or gradually reverted to virulence after inoculation into cattle\textsuperscript{22,109,118}. The second hypothesis suggests that mild strains for cattle emerge in cattle themselves in herds with intermediate levels of immunity. Maurer observed that RPV strains isolated from partially immune cattle herds usually had low virulence and produced mild gross lesions\textsuperscript{96}. Rossiter and James (1989)\textsuperscript{124} developed a model that, assuming longer incubation periods and less infectiousness, demonstrates that intermediate levels of immunity against RPV promote the establishment of endemicity of the infection and the circulation of mild rather than virulent strains.

It is not fully understood how highly virulent strains of morbillivirus species cause death, though the predilection of morbillivirus species for lymphoid tissues suggests part of the deaths are linked to immune failure. To increase understanding of virulence, characteristics of virulent wild-type strains have been compared to those of attenuated laboratory strains (mostly vaccine strains) and – in the case of RPV- to mild wild-type strains. For CDV, strains were also reverted to virulence to compare them to their attenuated laboratory parent\textsuperscript{5,149}. The results suggest that virulence of strains has an effect on latent and incubation periods, as well as on infectivity. First, in cattle, longer latent or incubation periods were observed in infections with attenuated\textsuperscript{139} or mild\textsuperscript{109} strains of RPV than in infections with virulent strains. Second, inoculation of attenuated laboratory strains or mild wild-type strains produced (up to a 1000-fold) lower viremia titers than inoculation with virulent wild-type strains (MV-monkey\textsuperscript{8,144}, RPV-cattle\textsuperscript{69,139}). Third, virulent and mild RPV wild-type strains were both lympho- and epitheliotrophic\textsuperscript{133,156}, whereas the Plowright RPV vaccine strain was strictly lymphotrophic and did not multiply in the mucosae of the respiratory and gastro-intestinal tracts\textsuperscript{139}. In infections with virulent or mild RPV wild-type strains, tissue alteration was closely associated with the presence of virus antigen, detected by immunohistochemistry. However, virulent RPV strains seemed to propagate faster and more extensively than mild strains, causing greater damage\textsuperscript{69,133,156}. Severe destruction and depletion of lymphocytes was seen with virulent RPV strains, whereas hyperplastic reactions were observed in the B- and T-cell areas of lymphatic tissues of cattle infected by mild RPV strains\textsuperscript{133}. Reversion to virulence of an attenuated CDV strain in dogs was paralleled by an increase in duration of lymphocytopenia and a reduction in lymphocyte blastogenesis\textsuperscript{5}.

To understand differences in virulence at the genetic level, genotypes of virulent strains...
have been compared to those of attenuated or mild strains (MV-human\textsuperscript{6,61}; RPV-cattle\textsuperscript{13}) and recombinant strains have been made, e.g., CDV-ferret\textsuperscript{149}. Loss or gain of virulence appears to involve multiple genes (RPV-cattle\textsuperscript{13}; CDV-ferret\textsuperscript{149}). For example, although the Plowright RPV vaccine strain has been shown to differ from the virulent virus from which it was derived by less than 0.55\%\textsuperscript{14,15}, the differences occur in all 6 genes of the genome\textsuperscript{13}. This domain of on-going research is not considered further in this paper.

**Infectiousness of the host, routes of excretion, and viral clearance**

The infectiousness of the host refers to the duration of the period when a host is infective on the one hand, and to the amount of an infectious agent that an animal can transmit on the other. The routes of excretion are the routes (sites) by which the virus leaves the infected host.

Consistent with the pathogenesis of morbillivirus infections, infective virus is found in blood, excreta –including expired air– and secreta, and skin scurf of infected hosts. Infectiousness varies per morbillivirus-host pair, and per individual. To detail the patterns of infectiousness in MV-human, RPV-cattle, CDV-dogs and CDV-ferret, the results of studies detecting infective virus by virus isolation on tissue culture, or by successful passage of the infection to susceptible test animals, are summarized in Figure 4 and the next paragraphs. The studies generally used onset of fever (RPV-cattle; CDV-dogs; CDV-ferrets) or onset of rash (MV-humans, i.e., approximately three days after the onset of fever\textsuperscript{26}) as temporal reference points for reporting the periods individuals were found to be infectious.

MV could generally be isolated from blood, throat, nasal aspirate and urine samples of infected humans for a few days around the onset of rash\textsuperscript{44,76,90,126,143} (Figure 4), and individual infectiousness could vary by at least one order of magnitude\textsuperscript{117}. Blood samples and nasal washings taken from human MV patients around the onset of rash induced lesions in the cynomolgus macaques into which they were inoculated. However, those taken four days after the onset of rash no longer did\textsuperscript{79}, infectiousness of MV-infected hosts usually ending abruptly shortly after the appearance of rash either because the host died, or because the host mounted an immune response (including neutralizing antibodies) that cleared the virus from tissues\textsuperscript{99}. Infectiousness lingered on in some hosts: such cases were either pregnant\textsuperscript{51}, had acute lymphoblastic leukemia\textsuperscript{31}, or developed (giant cell) pneumonia\textsuperscript{51,98} (Figure 4). Cases of subacute sclerosing panencephalitis (SSPE), a rare complication of a MV-infection that occurred years after initial infection, are not infectious. The brain contains defective virus\textsuperscript{61}.

Cattle inoculated with virulent RPV were most infectious around the 3\textsuperscript{rd} to 7\textsuperscript{th} day of fever\textsuperscript{28,87,151} (Figure 4). Blood, nasal exudate\textsuperscript{87,150,151} and ocular secretions\textsuperscript{101,150,151} were sometimes infectious before the onset of clinical signs. In cattle inoculated with mild wild-type RPV strains, the timing of viremia was either comparable to that of cases infected with virulent virus\textsuperscript{109} or occurred
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as a biphasic event with an initial first peak of infected PBMC occurring around days 2-5 post
inoculation (p.i.), and a second peak around days 9-12 p.i. Ind. Individuals varied in duration and
route of excretion. For example, of 24 head of cattle infected experimentally with virulent
RPV, 18 had virus in their nasal exudate for two or more days, 4 for one day, and 2 never did;
12 had virus in their urine for two or more days, 4 for one day, and 8 never did. The infectious
period of cattle infected with virulent RPV usually ended abruptly either by death or recovery,
the host’s immune response (including neutralizing antibodies) clearing the virus from tissues
(Figure 2). Most cattle that died were infectious until death, shedding large quantities
of virus. A few were successful in clearing the virus from their tissues before death. Experimentally infected cattle that recovered 10 days p.i. and was placed into contact with
susceptible cattle from the 11th to the 15th p.i., failed to spread RPV to these susceptible cattle.
The infectious period was sometimes extended in RPV-infected pregnant cows that seemingly
recovered but then aborted; the fetus could be infectious, and the cow itself could be viremic
(up to 21 days p.i.) or have infectious vaginal fluids (up to 34 days p.i.).

Dogs inoculated with virulent CDV generally developed fever approximately four days after
infection and were infectious for one or a few days. Subsequently, some dogs developed
neutralizing antibodies that reached protective titers from two weeks p.i.. These dogs rapidly
stopped shedding virus and recovered. In contrast, the other dogs had insufficient immune
response leading to a second bout of fever and severe respiratory and/or nervous signs. These
severely ill dogs generally died from three weeks up to two months p.i., shedding virus, albeit
intermittently, until their death. After their death, virus could be isolated from tissues like lung and brain. These terminal cases could thus extend the duration
of infectiousness, though irregular, up to two months. Cases of old dog encephalitis, a rare
complication of a CDV-infection that occurred years after initial infection, are not infectious.
Virus isolation is rarely successful from such cases, and although two studies report isolation of
non-defective virus, they did not exclude the possibility of recent re-infection.

Nasal exudate of ferrets inoculated with CDV by subcutaneous injection was infectious before
the onset of fever and other clinical signs. In ferrets infected by aerosol, blood contained virus
from the second day p.i. onwards. Skin scurf of CDV-infected ferrets inoculated into a ferret
induced infection and disease. In one study, urine recovered around the time of onset of fever
did not induce infection. Ferrets were very susceptible and usually died before neutralizing
antibodies appeared. In a related mustelid species, mink (Mustela vison), skin scurf scraped off
an infected animal at 25 days p.i. was infectious by inoculation to a ferret. Also, the pooled nasal
fluid from 10 infected mink was infectious by inoculation to ferrets from 5 days p.i. to 51 days
p.i., despite the clinical recovery of the mink around day 35 p.i. However, of the five mink still
alive on day 35 and seemingly recovered, all but one actually died with neurological signs within
100 days p.i., which suggests that—as for dogs—immune response to CDV infection had been
only partially sufficient.
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Measles in humans
A. Stages of disease (virulent virus) 61

B. Virus isolation on tissue culture
• from blood (on MK and HA cells) 106
• from throat (on MK and HA cells) 106
• from blood and nasal aspirates (on Vero and HA cells) 44
• from blood, throat or urine (on MK and HA cells) 90
• from blood (on lymphoblastoid cells) 766
• from throat swabs (on lymphoblastoid cells) 143
• from blood or hospital cases with pregnancy or pneumonia (on MK and HA cells) 51
• from nasopharynx of acute lymphoblastic leukemia patients 31
• from nasopharynx of MV giant-cell pneumonia cases (on HA and HK cells) 98

Rinderpest in cattle
A. Stages of disease (intranasally inoculated virulent virus) 110

B. Virus isolation on tissue culture or transmission to susceptible test animals
• from blood (on BK cells) 87
• from nasal exudate (on BK cells) 87, 151
• from ocular secretions (on BK cells or lymphoblastoid cells) 151
• in air (airborne transmission to susceptible bull) 75
• from urine (on BK cells) 87
• from feces (on BK cells) 87
• in blood or vaginal fluid of aborting cow/aborted fetus (inoculation to test animals) 78

Canine distemper in dogs
A. Stages of disease (aerosol virulent virus) 4

B. Virus isolation on tissue culture or samples infectious to test animals
• in blood of cases with mild disease (inoculation into ferrets) 119
• in blood of cases with mild disease (on FK cells) 57
• in urine (inoculation into dogs) 17
• in blood of cases with severe disease (inoculation into ferrets) 17, 19
• in blood of cases with severe disease (on FK cells) 20
• in blood of a severe case (inoculation into dogs) 17
• in urine of a CDV neurological case (inoculation into dogs) 17

Canine distemper in ferrets
A. Stages of disease (virulent virus, neurotropic) 98

B. Infectious to test animals
• in nasal secretions (inoculation into ferrets) 58
• in ocular secretions (inoculations into ferrets) 58
• in urine (inoculation into ferrets) 58
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**Figure 4:** Schematic representation of typical infections with virulent strains (MV-human$^{61}$; RPV-cattle$^{110}$, CDV-dog$^{4}$, CDV-ferret$^{58}$), with a compilation of study results on the duration of viremia and viral shedding via different routes for the morbillivirus-host pairs (MV-human$^{31,44,51,76,90,98,126,143}$; RPV-cattle$^{75,78,87,151}$; CDV-dog$^{17,29,119}$; CDV-ferret$^{58}$). Each square in the horizontal bars corresponds to a day.

The stages of disease: The dashed vertical line indicates the onset of the first clinical signs, the squares with the horizontal stripes days correspond to the incubation period, the grey squares and white squares the symptomatic period, the latter corresponding to the time period in which clinical signs start to disappear. The onset of fever is represented by a triangle, the appearance of rash (or time of widespread infection of non-lymphoid tissues) by a flower.

Virus isolation on tissue culture or samples infectious to test animals: The horizontal solid line indicates a period sampled daily, and the squares the days virus was found in at least one individual. When possible, differentiation was made into periods in which more (dark squares) or less (light squares) of the individuals were infectious. The horizontal dashed line indicates a period sampled irregularly, and the squares with crosses, days when at least one sample was positive. Abbreviations: MK=monkey kidney; HA=human amnion; BK=bovine kidney; FK=ferret kidney.
Individuals re-infected after having had a morbillivirus infection earlier in life or after being vaccinated may develop clinical disease or be subclinically infected, as outlined under host susceptibility (MV-humans\textsuperscript{47,100,147}; RPV-cattle\textsuperscript{10,114}; CDV-dogs\textsuperscript{104}). Cases of secondary MV vaccine failure (waning immunity after seroconversion) were not contagious\textsuperscript{47}. Most MV re-infections are diagnosed by serology\textsuperscript{47,100} and virus isolation or even detection of viral RNA by RT-PCR from naso-pharyngeal samples or urine are generally not successful\textsuperscript{88,132}, except in one report\textsuperscript{147}. In contrast, a single study reported that wild-type RPV was isolated from nasal exudate of cattle that had been vaccinated two years earlier with a tissue culture vaccine, had neutralizing antibody titers, and had been put into contact with two infectious animals. The vaccinated cattle showed no clinical signs other than ocular discharge for a day in a few of the animals, but fatally infected a susceptible animal housed with them\textsuperscript{114}.

Although host species that are less susceptible to a particular morbillivirus species tend to have subclinical infections, they may still be infectious. For example, sheep and goats, which tend to have subclinical RPV infections are infectious and can transmit the infection to susceptible in-contact cattle\textsuperscript{3,93,161}.

**Stability of the virus**

The stability of a virus is the length of time it can remain infective outside its host, or in a dead host.

Morbillivirus species, 0.15 to 0.3 µm in diameter, enter the environment in body fluids (blood, secreta and excreta) or in tissues of an infected carcass. The effects of temperature, pH and light on the stability of morbillivirus species are approximately defined by studies done in the context of the conditions for conservation of virus during storage and vaccine production. Deterioration at warm temperatures takes hours to days, whereas below freezing point morbillivirus species remain viable in various substrates for months to years; the very events of freezing and thawing, or the sequence, are detrimental (MV\textsuperscript{126}; RPV\textsuperscript{111}; CDV\textsuperscript{6}). The pH range for survival seems to be broad (RPV\textsuperscript{111}; CDV\textsuperscript{6}). Restricted studies on the effect of visible and UV light seem to indicate that these have an inactivating effect on virus in substrate. The speed of this process varies per substrate, proteineous substances slowing the rate of inactivation by light (MV\textsuperscript{34}; RPV\textsuperscript{111}; CDV\textsuperscript{102}).

The duration of virus stability in infected carcasses was examined, to our knowledge, only for RPV in cattle. Anecdotal evidence had suggested that RPV was inactivated in carcasses in a matter of hours in the tropics and of days in temperate climates\textsuperscript{33}. Subsequently, a study measured the half-life of wild-type and vaccine strains of RPV in lymph nodes, spleen and blood conserved at different temperatures. Independent of virus strain, half-life in lymph nodes and spleen was 5 minutes at 56°C, 105 minutes at 37°C, 6.4 hours at 25°C, and 2.3 days at 7°C, and 72
days at -15°C. The inactivation rate of RPV infected blood was similar to that of tissues at 56°C and 7°C, but 21 hours at 37°C, 1.5 days at 25°C.

The duration of virus stability in secreta and excreta was examined, to our knowledge, only for CDV-ferrets. At room temperature, nasal exudate and saliva on gloves used to handle ferrets still contained infective CDV particles after 20 minutes, but no longer after 30 minutes. Feces, food and water in uncleaned pens of ferrets removed from the cages after their death failed to convey CDV infection to susceptible ferrets, but cages from which moribund CDV infected ferrets were removed several hours before did, suggesting excreta from living animals remained infectious for at least several hours.

The duration of virus stability in the air was examined for MV and RPV. At temperatures of 20°C and 26°C respectively, the stability of MV and of RPV in air is the greatest at low (20-40%) relative humidity, intermediate at high (90%), and the lowest at intermediate relative humidity (60 –70%)(MV; RPV).

We note here that there is one report suggesting morbillivirus infection of mollusks.

Host behaviour

Host behaviour determines when conditions of sufficient exposure (route, duration, dose) are likely to occur in order to result in infection. Host behaviour can be altered by infection.

Knowledge of the general behaviour of a host species is useful for predicting situations that could favour the spread of morbillivirus infection, for example, situations in which susceptible hosts aggregate. Knowledge of normal host behaviour and social patterns is also useful for predicting situations in which latently infectious hosts may transmit morbillivirus infection.

However, most morbillivirus-infected hosts are diseased when they are infectious. Studies on morbillivirus-infected hosts showed their behaviour was altered by disease. Some simply lost their appetite (MV-monkey), had shortness of breath (MV-monkey), or isolated themselves (RPV-wildlife). Others became disoriented or aggressive. Disoriented behaviour occurred in RPV-infected kudu, transiently blinded due to corneal opacity, keratitis or both. Aggressive behaviour occurred in RPV-African buffalo (Syncerus caffer) and was associated with the extreme dehydration resulting from severe diarrhea. Aimless wandering, incoordination, aggressive behaviour and lack of fear occurred in raccoons (Procyon lotor) and grey foxes (Urocyon cinereoargenteus) infected with CDV, though not systematically, probably depending on the extent of involvement of the nervous system in the disease. Such disoriented or aggressive behaviour causes unusual movement patterns of severely infected hosts, and this can contribute to explaining introductions into new geographical areas, for example entire herds of...
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thirsty infected buffalo deviated from their usual migration routes in search for water.²⁰

Very little is known about how healthy susceptible hosts react to the diseased morbillivirus cases. The type of reaction could range from wariness and avoidance, through indifference, to curiosity or aggression, or concern and intense care.

Discussion

We used data from five morbillivirus-host pairs to examine how morbillivirus enters a host (the route of infection, the infectivity of the virus), develops in it (susceptibility of the host, virulence of the virus), and then leaves it (infectiousness of the host, routes of excretion) for the next host (stability of the virus, behaviour of hosts). There were elements of similarity and divergence among virus-host pairs for these eight factors. To conclude we use these to discuss possible implications for transmission of emerging marine morbillivirus diseases in general, and for transmission of phocine distemper virus (PDV) to and among harbour seals (Phoca vitulina) in particular.

Data on routes of excretion and infection pointed out behaviour likely to be associated with a high risk for transmission of infection. The data suggested that infected hosts excrete morbillivirus mainly via nasal, ocular and oral fluids and urine, though expired air, feces, reproductive organ fluids, blood and skin scabs can also contain infective virus. It supported the idea that the most likely site of infection for a new host is the respiratory tract including pharynx, be it by mode of direct contact (sniffing, droplet exposure) or indirect contact (breathing in virus in aerosol). Taken together, it suggested that interactive behaviour involving close contact of the head of the susceptible host with orifices of the infectious host, or droplets expelled from these is likely to be associated with a high risk of transmission of infection. Among harbour seals, such moments occur in the summer during breeding activities and play, mostly at water surface.³⁵,³⁶,⁴¹,¹¹⁵,¹³⁴,¹⁴⁵,¹⁵⁴. Alternatively, infection could occur without direct interaction between hosts, either by sniffing up virus contained in secreta and excreta, or by aerosol inhalation, although in open spaces this requires super-spreaders, or wind gusts (air movement that limits dilution). These events could occur on land or in water at the surface. Cetaceans expire and inspire with force,¹⁶ and when swimming in groups could inhale virus contained in expired blowhole secreta as droplets and aerosol. The data also indicated that there may be other sites of infection, in particular the intact eye (conjunctiva), the broken skin, the digestive tract and the urogenital tract. The latter two routes would not require the host to be on land or at the water surface. Behavioural studies both under normal conditions and during epidemics can shed light on the frequency of occurrence of different exposure situations, and may contribute to explaining heterogeneities in the infected populations.

Data on infective doses and infectiousness brought up some points relating to the length
Factors affecting morbillivirus transmission

of the latent and length and intensity of the infectious periods, important parameters in
disease models. Disease models have been developed for example to model the spread of PDV
epidemics\cite{39}, to evaluate the influence of epidemiological parameters and host ecology on this
spread\cite{66}, to predict the reoccurrence of PDV epidemics\cite{59,135}. Regarding the latent period, its
length for a given morbillivirus-host pair was modulated by the dose of infection. Regarding
the infectious period, it was generally short, usually a matter of a few days. It could start while
hosts were in the incubation period of the disease, and there was important individual variation
in infectiousness with existence of superspreaders. A recent study suggests that by account-
ing for such superspreaders in models, disease extinction becomes more likely, outbreaks rarer
but more explosive\cite{91}. Each morbillivirus-host pair had a few infected hosts who excreted virus
for longer, albeit intermittently, and generally not beyond two months. These were either sur-
viving pregnant individuals in which the disease spread to the foetus, individuals with certain
pre-existing immune deficiencies, or cases in which there was a delayed or partial immune
response to the infection and which developed into prolonged pneumonia cases or cases with
neurological complications.

It is well-recognized that morbillivirus species target the immune system and that co-infec-
tions aggravate disease and increase mortality. In two morbillivirus host pairs, another factor
was shown to aggravate disease and increase mortality: vitamin A deficiency. This is interest-
ing as environmental contaminants like PCBs may affect vitamin A levels in marine mammals.
Differences in vitamin A status may contribute to explaining to differences in harbour seal
mortality among different areas during PDV epidemics.

One of the big questions around the emerging marine morbilliviruses is where is the virus in
between epidemics. The data compiled showed that susceptibility to infection is different from
susceptibility to disease, as exemplified by grey seal (Halichoerus grypus) susceptibility to PDV
infection but not disease\cite{63}, but suggests that host species important in maintaining infection
in other morbillivirus-host pairs were susceptible to both infection and disease. As such, even
harbour seals should not be excluded as reservoirs for PDV. Some of the data presented here
suggests the size of the host population(s) required for maintaining the virus may be smaller
than expected based on the characteristics of the virus during epidemics: besides the possibil-
ity of cross-species transmission, there may be virus strains of lesser virulence, individuals with
extended infectiousness (more relevant to transmission when hosts are not aggregated in big
groups), or an out-of-host stage. Indeed, morbillivirus species are generally considered to be
short-lived outside of the host. However certain environments and seasons may combine condi-
tions which seem to favour virus stability. For example, during half the year the poles typically
combine low temperatures, little UV light, and low atmospheric humidity, conditions in which
infectiousness of substrates and carcasses could possibly be preserved for extended periods of
time. Such scenarios could be explored experimentally.
Factors affecting morbillivirus transmission

Reference List

Factors affecting morbillivirus transmission

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Stage-structured transmission of phocine distemper virus in the Dutch 2002 outbreak

Submitted

Laura W. Pomeroy • Petra Klepac • Ottar N. Bjørnstad • Thijs Kuiken • Albert D.M.E. Osterhaus • Jolianne M. Rijks

Department of Biology, Pennsylvania State University, USA; Center for Infectious Disease Dynamics, Pennsylvania State University, USA; Department of Entomology, Pennsylvania State University, USA; Department of Virology, Erasmus MC, Rotterdam, The Netherlands; Dutch Wildlife Health Center, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
Heterogeneities in transmission are crucial, since these events can dictate epidemic dynamics. To elucidate transmission patterns of phocine distemper virus (PDV) between harbour seals (*Phoca vitulina*) during the 2002 Dutch outbreak, we created three models to distinguish transmission dynamics. A model in which the host population exhibited strong heterogeneous mixing best described PDV dynamics (*p*=0.0004), indicating that stage-structured transmission occurs in the Dutch harbour seal population. To capture the stage transmission dynamics, we created a “who acquires infection from whom” (WAIFW) matrix solely from incidence data from seal strandings. Transmission between subadults and adults was very high, followed by transmission within the subadults. We confirmed the transmission estimates using the next-generation formalism to estimate $R_0$. The data produce quantitative transmission terms that can be used to describe roles of each stage class in the PDV outbreak; these findings can best be supported with harbour seal behavioural studies.

**Introduction**

Heterogeneities in transmission have long been recognized for shaping dynamics of infectious diseases. Heterogeneities can, for example, change invasion criteria\(^{18,32,52}\) and enhance spatial spread through superspreading\(^{28}\). Complex biological mechanisms usually underlie heterogeneities such as spatial geography, gender, age class, individual immunological differences in susceptibility or infectiousness, superspreading events, behavioural factors, genetic variation and other individual variation\(^{2,6,28,35}\). Several different approaches have been developed to account for such heterogeneities. For example, recognition of discrete classes permit the use of a more refined transmission term, $\beta_{ij}$, which captures the rate at which an infectious individual of class $j$ will infect a susceptible individual of class $i$\(^6\). The separate classes can encompass gender, age, stage, social, immunological, physiological, or behavioural differences. These detailed transmission rates are usually modelled using the “who acquires infection from whom” (WAIFW) matrix\(^{4,5,6,15,41}\).

While the WAIFW matrix has proven to be of great theoretical utility\(^{5,15,27,41}\), empirical approaches to estimation and characterization have often proven difficult because of lack of relevant data. Various efforts have employed contact tracing\(^{17,19}\) or inferred contacts\(^{16,25,49}\) to determine how a virus is transmitted through different classes in a population. In this paper, we investigate whether it is possible to estimate elements in the WAIFW matrix from detailed age or stage incidence data. We ask three nested questions: can we test the null hypothesis of homogeneous mixing from such data? How well can we identify the WAIFW elements from such data in the absence of more detailed contact tracing? Finally, can we compare our estimation method with the theoretical next-generation formalism to estimate $R_0$ in structured populations?
To address these questions, we investigate a case study of phocine distemper virus (PDV) in harbour seals (*Phoca vitulina*) in the Netherlands during the 2002 epidemic. PDV is a single-stranded, negative-sense RNA virus which is a member of the Morbillivirus genus, family *Paramyxoviridae*. In each individual, disease typically spans a two-week period, including both the latent and infectious disease stages. Mortality is high, partly due to co-infections.

Two outbreaks of PDV have affected seal populations throughout the entire North Sea region: the first outbreak occurred in 1988, in which 18,000 to 23,000 harbour seals died. This mass mortality event caused by the viral epidemic began on the Danish island of Ånholt on April 12, 1988 and ended within the calendar year. A second PDV outbreak occurred in 2002 with the same point of origin: initial cases of harbour seal stranding and mortality occurred on May 4, 2002. In this epidemic, approximately 22,000 to 30,000 harbour seals died, resulting in the largest recorded mass mortality event in marine mammals.

In the Netherlands, the first local case of PDV was found on June 16, 2002 on Vlieland and the local epidemic ceased at the end of November, as fully described elsewhere. In that time period, 2284 seals were stranded along the Dutch coast, including 2279 harbour seals and 5 grey seals. Interestingly, the timing of stranded seals showed age specificity. Not only was the index case a member of the subadult stage class, but the median stranding date of all subadults was significantly earlier than the median stranding date of both juvenile and adults. Together, the stranding data implicate stage-structured disease transmission and heterogeneous host mixing in the 2002 Dutch epidemic. Previous models describing the spread of PDV throughout the North Sea have assumed homogeneous mixing among different harbour seal age or stage classes. Subsequently, we inquire if it is possible to estimate the WAIFW matrix from the detailed incidence data and investigate the evidence for non-homogeneous mixing.

**Materials and methods**

Seals were classified into stages based on body length of stranded carcasses, since only some of the stranded seals were precisely aged by counting the cementum layers of a canine tooth. The juvenile class contained female seals less than 90 cm and male seals less than 95 cm. Subadults included females with body lengths between 90 cm and 120 cm and males with body lengths between 95 cm and 130 cm. Lastly, the adult category contained female seals with body lengths greater than 120 cm and males with body lengths greater than 130 cm. Using these body length classifications, the juvenile class contained most of the pups of the year, while the subadult class included most of the 1- and 2-year-old females and 1- to 3-year-old males. Finally, the adult class included most of the females older than 2 years and males older than 3 years.

The epidemic dynamics were captured with a susceptible-infected-removed (SIR) model,
Stage-structured transmission of phocine distemper virus in the Dutch 2002 outbreak

Let $b_{i,j}$ be the probability per unit time of disease transmission between a susceptible individual in demographic class $i$ and an infected individual in demographic class $j$. The transmission, or WAIFW matrix, is then a 3 by 3 matrix, $b = \begin{bmatrix} b_{1,1} & b_{1,2} & b_{1,3} \\ b_{2,1} & b_{2,2} & b_{2,3} \\ b_{3,1} & b_{3,2} & b_{3,3} \end{bmatrix}$. We considered three different transmission scenarios and built three different models. For the first model, we considered homogeneous mixing among different stage classes and equal transmission rates ($b_{i,j} = b$ for all $i,j$). For the second model, we assumed weak heterogeneous mixing among the host population: within-stage transmission – the diagonal of the transmission matrix – was allowed to differ from between-stage transmission, which was designated by the off-diagonal elements. This difference was scaled by a coefficient, $k$. The transmission matrix is then:

$$N = \begin{pmatrix} S & I & R \\ n_{1,1} & n_{1,2} & n_{1,3} \\ n_{2,1} & n_{2,2} & n_{2,3} \\ n_{3,1} & n_{3,2} & n_{3,3} \end{pmatrix}$$

Since the lifespan of harbour seals is much longer than the duration of the PDV outbreak, we assumed that population size did not change during the outbreak – except for deaths due to infection – and fixed the initial population size ($N$) at 5400 based on population censuses. For each element $n_{i,j}$ in the matrix, the subscript $i$ designates the stage-structure: the number 1 represents juveniles, the number 2 represents subadults, and the number 3 represents adults. Similarly, for each element $n_{i,j}$ in the matrix, the subscript $j$ designates the epidemic category: the number 1 represents susceptible individuals, the number 2 represents infectious individuals, and the number 3 represents removed individuals.

The matrix $N$ (Equation 1) was reorganized into a population vector by stacking the rows of the matrix. The population vector then designates all juveniles, all subadults, and all adults. Within each stage class, epidemic categories are designated as susceptible individuals, infected individuals, and removed individuals as in equation 2, where $'$ specifies a vector transpose.

$$n = \begin{pmatrix} n_{1,1} & n_{1,2} & n_{1,3} \\ n_{2,1} & n_{2,2} & n_{2,3} \\ n_{3,1} & n_{3,2} & n_{3,3} \end{pmatrix}'$$

Let $\beta_{i,j}$ be the probability per unit time of disease transmission between a susceptible individual in demographic class $i$ and an infected individual in demographic class $j$. The transmission, or WAIFW matrix, is then a 3 by 3 matrix, $\beta = \begin{bmatrix} \beta_{1,1} \\ \beta_{2,1} \\ \beta_{3,1} \end{bmatrix}$. We considered three different transmission scenarios and built three different models. For the first model, we considered homogeneous mixing among different stage classes and equal transmission rates ($\beta_{i,j} = \beta$ for all $i,j$). For the second model, we assumed weak heterogeneous mixing among the host population: within-stage transmission – the diagonal of the transmission matrix – was allowed to differ from between-stage transmission, which was designated by the off-diagonal elements. This difference was scaled by a coefficient, $k$. The transmission matrix is then:
For the third model, we assumed strong heterogeneous mixing between stages by allowing the transmission term to vary with the interactions within and between stages (Equation 4).

\[
\beta = \begin{pmatrix}
  k\beta_b & \beta_b & \beta_b \\
  \beta_b & k\beta_b & \beta_b \\
  \beta_b & \beta_b & k\beta_b
\end{pmatrix}
\]  

The transmission matrix (4) is symmetrical: \(\beta_{ij} = \beta_{ji}\). This property captures an assumption of the model: each contact event between two hosts results in a bi-directional transmission process, where the probability of the two hosts infecting each other is equal. Moreover, this assumes that differences in susceptibility or infectiousness among stage classes do not significantly impact transmission dynamics\(^6\).

Transmission probabilities were derived from the SIR model. The force of infection, \(\phi\), is the probability per unit time for a susceptible to become infected\(^{12}\). The recovery rate, \(\gamma\), is the inverse of the average latent plus infectious periods (\(\gamma = 1/14\)). The transitions between epidemic categories within a stage class is then:

\[
A_i = \begin{pmatrix}
  1 - \phi(n(t)) & 0 & 0 \\
  \phi(n(t)) & 1 - \gamma & 0 \\
  0 & \gamma & 1
\end{pmatrix}
\]

where \(\phi(n(t)) = 1 - \exp\left(-\sum_{i,j} \beta_{ij} n_{ij}(t)\right)\) (Equation 5).

Epidemic transitions for all three stage classes were given by the transition matrix \(A(n)\), in which the block diagonal matrices \(A_1\), \(A_2\) and \(A_3\) designate the epidemic transitions among juveniles, subadults and adults, respectively (Equation 6):

\[
A(n) = \begin{pmatrix}
  A_1 & 0 & 0 \\
  0 & A_2 & 0 \\
  0 & 0 & A_3
\end{pmatrix}
\]  

Since we assumed that the epidemic dynamics are fast relative to demography, there are no transitions between stage classes, and the remaining elements of the block-diagonal matrix \(A(n)\) are all zero. The epidemic trajectory is given by multiplying the population vector at time \(t\), \(n(t)\), with the transition matrix \(A(n)\):

\[
n(t+1) = A(n(t))n(t)
\]  

This model (7) is a discrete time approximation to the continuous time model:
Because of the underlying stage-structure there are three infected classes in this model. For models with multiple classes, $R_0$ can be derived using the next generation method\textsuperscript{10,12,13,48}, where $R_0$ is given by the spectral radius, $\rho$, or the dominant eigenvalue of the next generation matrix, $FV^{-1}$:

\[
R_0 = \rho \left[ FV^{-1} \right]
\]

The rate of change of compartment $i$ is then

\[
\frac{dn_i}{dt} = F_{i}(n) - V_i(n) \tag{11}
\]

where $i,j = 1,\ldots,r$ and $n_0$ was the disease-free equilibrium, at which the population remains in the absence of the disease\textsuperscript{48}. The $(j,k)$ entry of $V^{-1}$ is the average amount of time an infective individual that was introduced into compartment $k$ spends in compartment $j$ during its lifetime. The $(i,j)$ entry of $F$ is the rate at which infected individuals in compartment $j$ produce new infections in compartment $i$. Therefore, the entry $(i,k)$ in the generation matrix $FV^{-1}$ is the expected number of new infections in compartment $i$ produced by an individual originally introduced into compartment $k$.

The matrix $F$ shows the influx of new infections to the infectious compartments. Since we assumed that there are no transitions between the infectious classes due to growth during this acute PDV outbreak, matrix $V$ reflects the rates at which individuals are leaving the infectious compartments due to recovery or death. At the disease-free equilibrium the population consists wholly of susceptible individuals, so that

\[
n_0 = \begin{bmatrix} n_{11} (0) & 0 & 0 & n_{21} (0) & 0 & 0 & n_{31} (0) & 0 & 0 \end{bmatrix}'
\]
Stage-structured transmission of phocine distemper virus in the Dutch 2002 outbreak

F and V were constructed as follows:

\[
F = \begin{pmatrix}
\beta_{11}n_{11}(0) & \beta_{12}n_{11}(0) & \beta_{13}n_{11}(0) \\
\beta_{21}n_{21}(0) & \beta_{22}n_{21}(0) & \beta_{23}n_{21}(0) \\
\beta_{31}n_{31}(0) & \beta_{32}n_{31}(0) & \beta_{33}n_{31}(0)
\end{pmatrix}
\quad \text{and} \quad
V = \begin{pmatrix}
\gamma & 0 & 0 \\
0 & \gamma & 0 \\
0 & 0 & \gamma
\end{pmatrix}
\]

The next generation matrix is thus:

\[
FV^{-1} = \begin{pmatrix}
\beta_{11}n_{11}(0) & \beta_{12}n_{11}(0) & \beta_{13}n_{11}(0) \\
\gamma & \gamma & \gamma \\
\beta_{21}n_{21}(0) & \beta_{22}n_{21}(0) & \beta_{23}n_{21}(0) \\
\gamma & \gamma & \gamma \\
\beta_{31}n_{31}(0) & \beta_{32}n_{31}(0) & \beta_{33}n_{31}(0) \\
\gamma & \gamma & \gamma
\end{pmatrix}
\]

\(R_0\) is given by the dominant eigenvalue of the next generation matrix (13). We determined \(R_0\) using equation 13 and our estimate of \(\beta\).

Initial model conditions for the total population size (N), recovery rate, gamma – the inverse of the latent period – population stage structure, and the length of the epidemic were derived from the literature (Table 1). The three models – homogeneous mixing, weak heterogeneous mixing, and strong heterogeneous mixing – were compared using the likelihood ratio test (LRT). Subsequently, p-values were calculated to determine which model best fits the data.

**Table 1:** Initial Model Conditions. Initial conditions for the PDV SIR model were obtained from the literature. Total population size (N) was estimated for the entire Dutch harbour seal population, including seals that are on land and in the water at any given time.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (total population size)</td>
<td>5400</td>
<td>39</td>
</tr>
<tr>
<td>Latent and Infectious Periods</td>
<td>14 days</td>
<td>44</td>
</tr>
<tr>
<td>(\gamma) (inverse of the latent and infectious periods)</td>
<td>1/14</td>
<td>44</td>
</tr>
<tr>
<td>Population Stage Structure</td>
<td>15% juveniles</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>36% subadults</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49% adults</td>
<td></td>
</tr>
<tr>
<td>Length of Epidemic</td>
<td>180 days</td>
<td>40</td>
</tr>
</tbody>
</table>

To obtain estimates for the WAIFW matrix, we used maximum likelihood techniques to find the values of the matrix elements which best fit the stage-specific incidence data and the probability of observing a stranded seal, \(p\) (14). The probability of observation is a compound
variable encompassing both the probability that a given seal, once infected by PDV, will strand and that the stranded seal will be encountered and observed. We assume Poisson likelihoods for disease incidence.

\[ y \sim Po(\lambda) \]  

(14)

Data for this model consisted of stranded seals from the Dutch islands of Vlieland, Terschelling, Ameland, Schiermonnikoog, and Texel, and from the mainland provinces of Friesland, Groningen, and Noord Holland. Point estimates were located by minimizing the negative log-likelihood of the data using simulated annealing\(^8\) as implemented by the ‘optim’ function in R\(^{36}\). Strong colinearity between elements in the WAIFW matrix led to a range of near equally likely results, since values for elements of the WAIFW matrix can compensate for each other to produce the same results in disease incidence. This colinearity was investigated by inverting the numerical Hessian matrix and converting variance-covariance matrix into the corresponding correlation matrix\(^30\). If the pair-wise correlation coefficient between two elements was greater than 0.3, we used two-dimensional profile likelihoods to map plausible pair-wise parameter combinations including their two-dimensional 95% confidence intervals. We tested the significant deviation from homogeneous and weak heterogeneous mixing using likelihood ratio tests (LRTs).

All model building and parameter estimations were performed using R version 2.3.1\(^{36}\). The next-generation estimates of \(R_0\) were performed using Mathematica version 6\(^{51}\).

**Results**

The data were stratified by stage class (Figure 1). The three models created were fitted to the data and compared using the likelihood ratio test (LRT). The first model, homogeneous mixing with uniform \(\beta\), implies complete lack of stage structure in the population. Results from the model selection tests (Table 2) show that the model with slight heterogeneous mixing has a better fit to the data than the model with homogeneous mixing (\(p = 0.001\)). The strong heterogeneous mixing model fit the data better still (\(p = 0.0004\)) (Table 2). When comparing the set of the three nested models, the best-fit model overall was the model with the strong heterogeneous mixing which permitted unique within-and between-stage interactions (Table 2).

Using the model with strong heterogeneous mixing, chosen by the model selection test, we estimated point values for each of the elements in the WAIFW matrix according to the maximum likelihood estimates (Table 3). For the juvenile stage, transmission with subadults comprised the greatest component of disease incidence (\(\beta_{12} = 9.09 \times 10^{-3}\)), closely followed by transmission within the juvenile stage (\(\beta_{11} = 5.56 \times 10^{-3}\)) and transmission between juveniles
Figure 1: Temporal stranding of harbour seals. The total number of harbour seals stranded and number of harbour seals stranded stratified by stage class are shown for each week of the PDV epidemic. The first week began in mid-June while the last cases were counted at the end of November.

Table 2: Model selection using the likelihood ratio test (LRT). Three models, used to describe the spread of PDV in the Dutch Wadden Sea, were compared to see which best fit the data. These models include: homogeneous mixing with uniform $\beta$, slight heterogeneous mixing with $\beta_1$ and $k\beta_2$, and the full stage-structure model with the symmetrical $\beta$ matrix. The likelihood ratio, degrees of freedom, and p-values are listed for each pair-wise model comparison. Overall, the model incorporating full stage structure and symmetrical $\beta$ matrix was the best-fit model to the data.

<table>
<thead>
<tr>
<th>Models</th>
<th>Likelihood Ratio</th>
<th>Degrees of Freedom</th>
<th>P-value</th>
<th>Better-fit Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous mixing</td>
<td>5.28</td>
<td>1</td>
<td>0.00116</td>
<td>Slight heterogeneous mixing</td>
</tr>
<tr>
<td>Slight heterogeneous mixing</td>
<td>10.14</td>
<td>4</td>
<td>0.0004385</td>
<td>Strong heterogeneous mixing</td>
</tr>
</tbody>
</table>

and adults ($\beta_{13} = 5.05 \times 10^{-6}$) (Table 3). Intra-stage transmission ($\beta_{11}$) and transmission between juveniles and subadults ($\beta_{12}$) provided clear maximum likelihoods with targeted values; the point estimate for transmission between juveniles and adults ($\beta_{13}$) was less obvious since it lies within a range of equally likely results. Subadults, in contrast, showed the greatest interaction with members of their own class ($\beta_{22} = 3.74 \times 10^{-4}$), followed by interactions with adults ($\beta_{23} = 2.22 \times 10^{-4}$) and then juveniles ($\beta_{21} = 5.56 \times 10^{-5}$) (Table 3). Again, intra-stage transmission ($\beta_{22}$) and transmission between subadults and juveniles ($\beta_{21}$) provided clear estimates; the point estimate for transmission between subadults and adults ($\beta_{23}$) was more obscure due to the fact that it lies within a range of results with similar likelihoods. Lastly, adult transmission was greatest with subadults ($\beta_{32} = 2.22 \times 10^{-4}$) and decreased with both adults ($\beta_{33} = 8.08 \times 10^{-5}$) and juveniles ($\beta_{31} = 5.05 \times 10^{-6}$) (Table 3). The transmission parameters for adults demonstrated the greatest degree of uncertainty among the three stages: each adult transmission term falls within a range of equally likely values.
Table 3: Parameter point estimates in the full stage structure model from one-dimensional likelihoods. Point estimates and 95% confidence intervals for each element in the symmetrical $\beta$ matrix in the model incorporating full stage structure were obtained by maximum-likelihood methods. The intra-stage transmission terms are designated by $\beta_{1,1}$ for juveniles, $\beta_{2,2}$ for subadults, and $\beta_{3,3}$ for adults. Inter-stage transmission is symmetrical. Interactions between juveniles and subadults are designated by $\beta_{1,2} = \beta_{2,1}$, while transmission between juveniles and adults are designated by $\beta_{1,3} = \beta_{3,1}$. Finally, the transmission between subadults and adults is designated by $\beta_{2,3} = \beta_{3,2}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Demographic Classes</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_{1,1}$</td>
<td>Juvenile – Juvenile</td>
<td>$5.56 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_{2,2}$</td>
<td>Subadult – Subadult</td>
<td>$3.74 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\beta_{3,3}$</td>
<td>Adult – Adult</td>
<td>$8.08 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_{1,2} = \beta_{2,1}$</td>
<td>Juvenile – Subadult</td>
<td>$9.09 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_{1,3} = \beta_{3,1}$</td>
<td>Juvenile – Adult</td>
<td>$5.05 \times 10^{-6}$</td>
</tr>
<tr>
<td>$\beta_{2,3} = \beta_{3,2}$</td>
<td>Subadult – Adult</td>
<td>$2.22 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

The ambiguity in point estimates reflects the high degree of colinearity among the stage-structured parameter point estimates in Table 3. In other words, the likelihood landscape is rugged: there are different combinations of parameter values that could result in equally fit likelihood values. Correlations between parameters, calculated from the unconstrained optimization of all parameters in the full stage-structure model, are shown in Table 4. Two variables are considered to be correlated if their correlation coefficient was greater than 0.3. Under this criterion, four sets of variables are highly correlated: intra-stage juvenile transmission ($\beta_{1,1}$) and transmission between juveniles and subadults ($\beta_{1,2} = \beta_{2,1}$), intra-stage subadult transmission ($\beta_{2,2}$) and transmission between juveniles and subadults ($\beta_{1,2} = \beta_{2,1}$), intra-stage adult transmission ($\beta_{3,3}$) and transmission between juveniles and adults ($\beta_{1,3} = \beta_{3,1}$) and, finally, intra-stage adult transmission ($\beta_{3,3}$) and transmission between subadults and adults ($\beta_{2,3} = \beta_{3,2}$) (Table 4).

Table 4: Correlation coefficients in the full stage structure model from the unconstrained optimization of all parameters. Hessian matrix and corresponding correlation matrix was calculated from the unconstrained optimization of all parameters in the model incorporating full stage structure simultaneously. Both negative and positive correlations are identified. Correlations greater than 0.3 are highlighted.

<table>
<thead>
<tr>
<th></th>
<th>$\beta_{1,1}$</th>
<th>$\beta_{2,2}$</th>
<th>$\beta_{3,3}$</th>
<th>$\beta_{1,2} = \beta_{2,1}$</th>
<th>$\beta_{1,3} = \beta_{3,1}$</th>
<th>$\beta_{2,3} = \beta_{3,2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_{1,1}$</td>
<td>1.00</td>
<td>0.0201</td>
<td>0.0158</td>
<td>-0.842</td>
<td>-0.0239</td>
<td>-0.00847</td>
</tr>
<tr>
<td>$\beta_{2,2}$</td>
<td>0.0201</td>
<td>1.00</td>
<td>-0.00818</td>
<td>-0.506</td>
<td>0.00953</td>
<td>-0.00649</td>
</tr>
<tr>
<td>$\beta_{3,3}$</td>
<td>0.0158</td>
<td>-0.00818</td>
<td>1.00</td>
<td>0.0484</td>
<td>-0.853</td>
<td>-0.312</td>
</tr>
<tr>
<td>$\beta_{1,2} = \beta_{2,1}$</td>
<td>-0.842</td>
<td>-0.506</td>
<td>0.0484</td>
<td>1.00</td>
<td>-0.0827</td>
<td>-0.0337</td>
</tr>
<tr>
<td>$\beta_{1,3} = \beta_{3,1}$</td>
<td>-0.0239</td>
<td>0.00953</td>
<td>-0.853</td>
<td>-0.0827</td>
<td>1.00</td>
<td>0.0689</td>
</tr>
<tr>
<td>$\beta_{2,3} = \beta_{3,2}$</td>
<td>-0.00847</td>
<td>-0.00649</td>
<td>-0.312</td>
<td>-0.0337</td>
<td>0.0689</td>
<td>1.00</td>
</tr>
</tbody>
</table>
The basic reproductive ratio, as given by the dominant eigenvalue of the next generation matrix (Equation 12) using transmission estimates from the unconstrained simultaneous optimization of all parameters, gave an estimate of $R_0 = 3.08$, which falls directly in the range of other $R_0$ estimates for PDV\textsuperscript{43}.

Discussion

Age-or stage-structured behaviour dictates transmission of many diseases\textsuperscript{3,4,5,41} and evidence points to stage-structured PDV transmission in harbour seals. For example, harbour seals show characteristics of discriminate interactions based on stage class\textsuperscript{20,37,38,42,45,50} and PDV incidence has previously been shown to have signatures of stage dependence in the Dutch 2002 outbreak\textsuperscript{40}. In this paper, we develop a theoretical framework to incorporate stage structure in PDV epidemic SIR models. By creating and ranking three nested models ranging from a complete lack of stage structure with homogeneous mixing to strong heterogeneous mixing, we showed that added stage structure provided a better description of the data (Table 2). Overall, the model with strong heterogeneities was the best-fit model (Table 2), indicating that the harbour seal population in the Dutch Wadden Sea transmitted PDV in a stage-dependent manner during the 2002 epidemic.

Using the full stage-structure model with the symmetrical $\beta$ matrix, we were able to determine elements of the WAIFW matrix from incidence data alone (Table 3), illuminating both mechanisms of epidemic spread and harbour seal contact structure. The highest transmission occurs among the subadult class and between subadults and adults, while the lowest transmission occurs between juveniles and adults. Other combinations of stage classes show intermediate levels of transmission. We were able to verify our estimates by calculating $R_0$ using the next-generation matrix, which resulted in a value that is acceptable for PDV ($R_0 = 3.08$).

Among all of the classes for which elements of the WAIFW matrix have been defined, subadults emerge as an interesting and vital category. Since the index case and most early cases were in the subadult category\textsuperscript{40}, it logically follows that subadult-subadult transmission would be high since there are many behavioural contacts that may be epidemiologically relevant.

However, the elements in the WAIFW matrix is highly correlated, so it is difficult to unambiguously estimate certain pairs of parameters (Table 4). For example, transmission between juveniles and subadults are highly correlated with both intra-stage juvenile transmission and intra-stage subadult transmission. This makes it difficult to distinguish between an intra-stage transmission chain and a “pathogen rain” that infects the juvenile class from many other stages. Another reason for the correlation may stem from the fact that the stage classes were based on a proxy for age, namely sex and body length. Exact age determination may have lessened the correlations. Nevertheless, correlation between elements
of the WAIFW matrix set limits to the identifiability of the matrix. Encouragingly, though, likelihood ratio tests appear to have an ability to distinguish heterogeneous from homogeneous mixing in stage-structured data.

The WAIFW matrix is a catalogue of “who acquires infection from whom;” therefore, it is a reflection of transmission events and not a direct reflection of the contact process among healthy or immune seals. For instance, the stage class that introduces the disease into the population appears to influence the strength of the transmission coefficients. Many early cases in the 2002 Dutch PDV epidemic were subadults$^{40}$; this is reflected in the high transmission between juveniles and subadults, among subadults, and between adults and subadults (Table 3). In addition, seals may have altered their behaviour due to disease. Nevertheless, the resulting transmission rates also seem to a mirror harbour seal behaviour under normal conditions. For example, from point estimates, juvenile transmission is greatest with subadults and decreases with other juveniles and adults (Table 3). The juveniles stranded in September, and therefore generally more than two weeks after weaning. At this time they are no longer in relative isolation with their mothers$^{20,31,46}$.

Although many insights on PDV transmission between harbour seal stage classes in the Dutch Wadden Sea have been gained by the incidence data and resulting WAIFW matrix, the ambiguity in the estimates highlights the need for additional behavioural data. To make the theoretical results relevant, information about the number and type of intra-stage and inter-stage interactions must be determined. For example, specific contact measurements between and among stage classes could be determined based on the distance between individuals from observations or aerial photographs. In particular, focusing on the behaviour of the subadult stage would be the most helpful since the index case fell into this category. These additional behavioural data will likely constrain the degree of uncertainty in the parameter estimates to biologically reasonable and relevant ranges.

In conclusion, elements in the WAIFW matrix that provide information about transmission dynamics within and between stage classes of harbour seals can be estimated based on stage incidence data alone. Combining our statistical methodology with the next-generation formalism further allows us to estimate $R_0$. However, identifiability and uncertainty issues still exist within these parameter estimates, highlighting the need for additional behavioural data to restrict the ranges of the theoretical parameter estimations to biologically plausible and realistic values. Stage structure clearly plays an important role in the dynamics of this epidemic.
**Acknowledgements**

The authors would like to thank Bryan Grenfell for his assistance with both theory and analysis, Michael Neubert and Hal Caswell for help with the model formulation, and Angela Luis for helpful discussions. In addition, the authors wish to thank the volunteers who covered the Dutch coast daily in search of stranded seals; the people working at the Seal Research and Rehabilitation Center in Pieterburen for logistical support; V.O.P. Containers for providing the location to necropsy the seals; the staff of the Dutch Ministry of Agriculture, Nature and Food Quality (LNV-Noord) for providing access to and help with the centralized seal registration data; the Common Wadden Sea Secretariat and the Trilateral Seal Expert Group for international coordination of the outbreak. Laura Pomeroy was supported by the National Science Foundation, under the NSF Graduate Teaching Fellowship in K-12 Education (DGE-0338240).
Reference List


Stage-structured transmission of phocine distemper virus in the Dutch 2002 outbreak


3

Pathology
3.1

Quantitative analysis of the 2002 phocine distemper epidemic in The Netherlands

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Jolianne M. Rijks • Fiona L. Read • Marco W.G. van de Bildt • Hester G. van Bolhuis • Byron E.E. Martina • Jaap A. Wagenaar • Karst van der Meulen • Albert D.M.E. Osterhaus • Thijs Kuiken

Department of Virology, Erasmus MC, Rotterdam, The Netherlands; Dutch Wildlife Health Center, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; Veterinary Medicine Diagnostic Center, Utrecht University, Utrecht, The Netherlands; and Seal Rehabilitation and Research Center Lenie ’t Hart, Pieterburen, The Netherlands.
Phocine distemper virus (PDV) caused thousands of deaths among harbour seals (*Phoca vitulina*) from the North Sea in 1988 and 2002. To examine the effects of different factors on the pathology of phocine distemper, we performed necropsies and laboratory analyses on 369 harbour seals that stranded along the Dutch coast during the 2002 PDV epidemic. Diagnostic tests for morbillivirus infection indicated a differential temporal presence of morbillivirus in lung and brain. Seals of 3 years or older were significantly more often IgG positive than younger seals. The most frequent lesions in PDV cases were bronchopneumonia, broncho-interstitial pneumonia, and interstitial emphysema. Extra-thoracic emphysema was rare in <1-year-olds compared with older seals, even though severe pneumonia was more common. PDV cases generally had empty stomachs and less blubber than by-caught seals from before the epidemic. In PDV cases involving older animals, lung, kidney, and adrenal weights were significantly increased. *Bordetella bronchiseptica* was isolated from lungs in two thirds of the PDV cases examined. Our results indicate that brain should be included among the tissues tested for PDV by RT-PCR; that either phocine distemper has a longer duration in older seals or that there are age-related differences in immunity and organ development; that dehydration could play a role in the course and outcome of phocine distemper; and that bacterial coinfections in lungs are more frequent in PDV cases than gross lesions suggest. These results illustrate how quantitative analysis of pathology data from such epidemics can improve understanding of the causative disease.

**Introduction**

Phocine distemper virus (PDV), a morbillivirus affecting marine mammals, caused thousands of deaths in the harbour seal (*Phoca vitulina*) populations of the North Sea in 1988 and again in 2002. The PDV epidemics occurred in the summer, during the harbour seal birth, molt and breeding season. At this time of the year, harbour seals show relative site fidelity, hauling out on (mostly intertidal) sandbanks in the Wadden Sea (3,600 counted in 2001) or in the Delta area (150 counted in 2001). Pups are born between the end of May and the beginning of July, and weaned approximately 24 days after birth. The mating season is from July to August. The exact timing of molt during the summer varies with seal age and sex. During molt and breeding activities, seals may partly or completely reduce their food intake. Besides harbour seals, there were approximately 500 grey seals (*Halichoerus grypus*) in the Wadden Sea, hauling out mainly on a sandbank between Terschelling and Vlieland. PDV-associated deaths were not observed in this species.

During both epidemics, seal carcasses that stranded along the North Sea coasts during the two epidemics were necropsied, and the pathology of PDV was described in harbour seals from the UK, Denmark, Sweden, and Germany. Broncho-interstitial pneumonia, interstitial pneumonia, or suppurative bronchopneumonia, and lymphocytic depletion in lymphoid tissues were the most
consistent lesions. Non suppurative encephalitis, sometimes with demyelination, was found occasionally. Coinfections with parasites (lice, worms) and bacteria (in particular *Bordetella bronchiseptica*) were common\(^{3,7,25,28,38,39}\).

Up to now, the pathology of seals stranding along the Dutch coast during the PDV outbreaks has not been described. Moreover, although the pathology of phocine distemper has been described in general, many aspects are poorly understood. For example, we do not know the effect of storage method or choice of tissue on the outcome for morbiliavirus diagnostic assays; the effect of seal age on the character of the lesions or the outcome of diagnostic tests; the relationship between PDV infection and nutritional state; or the relationship between bacterial coinfections and the observed lesions.

The aim of this study was therefore to describe the pathology of PDV in harbour seals that stranded in The Netherlands in 2002, and to increase our understanding of the pathology of PDV infection in harbour seals in general by a quantitative approach to questions, such as those mentioned in the previous paragraph. We used a sample of 369 seals that were necropsied during the 2002 PDV epidemic in The Netherlands. Morbiliavirus diagnostic assays were performed on these seals and effects of storage method and sample type assessed. Excluding the 137 non-confirmed cases, we then used the 232 confirmed cases for describing lesions, state of nutrition, and coinfections, and for determining correlations between assay results, lesions, age, and coinfections. A subset of 29 confirmed PDV cases was used for histologic analysis and a subset of 36 for bacteriologic analysis. The systematic collection of seal carcasses and the consistent necropsy procedure applied before and during the outbreak provided the opportunity to compare organ weights, nutritional state, and bacterial coinfections of PDV-infected seals with those of pre-epidemic seals that died of other causes.

**Materials and Methods**

*The PDV epidemic sample*

More than half of the 2284 seals that stranded along the Dutch coast during the 2002 phocine distemper epidemic were necropsied. Most carcasses were stored in –20°C freezing containers placed on the Dutch Wadden Sea islands or the mainland for the duration of the epidemic. Several mass necropsy sessions were organized to examine these carcasses, subsequently referred to as ‘frozen seals’. In a minority of cases, necropsy was performed soon after death or euthanasia of the seal and these carcasses were stored at +4°C until necropsy. These are referred to as ‘cooled seals’ in this paper.

Life history data were collected from all carcasses. Pathologic information and biological samples were collected almost exclusively from carcasses in which the organs had retained their shape and most of their colour (fresh or mildly decomposed carcasses). Seals taken into
rehabilitation before their death were excluded. The final number of seals from the 2002 PDV epidemic discussed in this paper consisted of 369 harbour seals (54 cooled, 315 frozen).

**Diagnostic assays for morbillivirus**

Samples from the carcasses of the 369 seals were tested by at least one and up to four assays to detect morbillivirus infection. The samples were taken as described in Kuiken and Baker (1991)\(^3\).

First, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect morbilliviral RNA in lung tissue of 361 (51 cooled, 310 frozen) seals, as described elsewhere\(^2\). Kidney, urinary bladder, and brain tissues were also tested in cooled seals, and kidney and urinary bladder tissues in a number of frozen seals. The samples were preserved in lysis buffer (6 M Guanidine-HCL, 10 mM Urea, 10 mM Tris-HCl, 20 % Triton-X100 (v/v), pH 4.4) and stored at −20°C or room temperature until analysis.

Second, an indirect avidin–biotin–complex immunohistochemistry (IHC) test was performed to detect morbillivirus antigen in formalin-fixed, paraffin-embedded tissues of 83 seals (38 cooled, 45 frozen), as described elsewhere\(^15,21,28\). The primary antibody used was polyclonal rabbit anti-meades virus serum. The tissues tested were lung, spleen, liver, kidney, urinary bladder for all seals, and additionally cerebrum and cerebellum for the cooled seals.

Third, serum samples of 82 seals (46 cooled, 36 frozen) were tested for morbillivirus-specific immunoglobulin M (IgM) antibody by an antibody-capture enzyme-linked immunosorbent assay (ELISA). Plates were coated with goat anti dog-IgM for capturing IgM, and horseradish-peroxidase–labeled CDV antigen was used to detect bound IgM\(^3\). Serum samples were centrifuged at 10,000 g for 5 min, and stored at −20°C until use.

Finally, the sera of 326 seals (53 cooled, 273 frozen) were tested for morbillivirus-specific immunoglobulin G (IgG) antibodies by indirect ELISA\(^4\). Plates coated with CDV antigen were incubated with seal serum and subsequently with horseradish-peroxidase–conjugated protein A to detect bound IgG. Sera were centrifuged and stored as previously described for IgM serology.

Though these assays were specific for morbillivirus rather than for PDV, it had previously been established that PDV was the morbillivirus causing the epidemic\(^2\). Cases positive in one or more of these four assays are referred to as ‘PDV cases’ in this paper.

**Necropsy findings in PDV cases**

Necropsies followed a standard protocol\(^3\). Body length was measured as the straight distance from the nose tip to tail tip measured alongside the carcass with the carcass lying flat on the
back. Blubber thickness was measured half-way along the length of the sternum. Pneumonia was called extensive when more than 50% of the lung volume was estimated to be firm. The lungs, liver, heart, and spleen of 35 cooled seals (17 males, 18 females) were weighed as were the kidneys, brain, and adrenals of 34 of these. For logistic reasons, organs were not weighed and brains were not examined in the frozen seals.

Formalin-fixed, paraffin-embedded samples of lung, spleen, kidney, urinary bladder, liver, cerebrum, cerebellum, adrenal, and occasionally other tissue sections of 29 cooled PDV cases were sectioned at 5 μm, stained with hematoxylin and eosin (HE) following standard procedures, and examined by light microscope. Additionally, lung tissue sections of 131 frozen PDV cases were stained (HE) and examined for numbers of neutrophils.

The age of a seal was determined by counting the number of cementum layers in one of the upper canine teeth. Three age classes were used in the paper: <1-year-olds, 1- and 2-year-olds, and 3-year-olds and older. The <1-year-olds are pups, 1- and 2-year-olds are subadults, and 3-year-olds and older are mostly adults (F. Read, personal communication).

Tissues were screened for coinfections with phocine herpes virus 1, bacteria, worms, and lice (Echinopthirius roddius). To detect coinfection with phocine herpes virus 1, lung tissue samples preserved in lysis buffer of 169 frozen seals were tested by PCR. Bacterial culture was performed on lung and tracheo-bronchial lymph node samples of 36 seals (26 cooled, 10 frozen); on liver, kidney, and spleen samples in 26 cooled seals; and on samples of other organs if gross lesions suggested that these were infected with bacteria. The samples were kept cool at +4°C until culture on aerobic blood agar, anaerobic blood agar, and Mac Conkey agar, and additionally on blood agar with increased CO2 for Brucella sp. In the 26 cooled seals, external iliac lymph node, and the uterus or testis samples were also cultured specifically for Brucella sp. Finally, lung-, heart- and stomach-worm burdens and numbers of lice were estimated and recorded as one of four levels (0; 1–10; 11–100; >100) in both cooled and frozen seals. Worms from cooled seals were fixed in 70% ethanol for identification.

The harbour seals used for comparison with PDV cases: the non-PDV cases Organ weights, state of nutrition, and bacteriology results of PDV cases were compared with those of nonepidemic seals stranded dead on the coast of The Netherlands. These are referred to as 'non-PDV cases' in this paper.

For organ weights, the comparison group consisted of all 16 nonepidemic seals for which organ weights were available. The deaths of these 16 seals were associated with by-catch and other accidents (n = 6), mesenteric torsion (n = 2), lung- or heartworm infection (n = 2), bacterial infection (n = 3), or undetermined cause (n = 3). The weights of lung and heart were recorded for all 16 cases, liver and spleen in 15 of them, kidney in 12, adrenals in 8, and brain in 6.
For state of nutrition, the comparison group consisted of all 25 non epidemic seals for which stomach contents and blubber thickness had been recorded and that had stranded in the same time period as the epidemic (16 June to 29 November) in one of the two years preceding the epidemic (2000 and 2001). The last two criteria were applied to minimize the influence of seasonal and between-year variation on the results. The deaths of the 25 seals were associated with by-catch \( n = 5 \), foreign body \( n = 2 \), mesenteric torsion \( n = 2 \), lung- or heartworm infection or both \( n = 3 \), bacterial infection \( n = 4 \), or undetermined cause \( n = 9 \).

For bacteriologic results, the comparison group consisted of all eight seals that stranded in the time period detailed in the previous paragraph and from which specific bacteria had been cultured. The deaths of these eight cases were associated with a flipper or prostate abscess \( n = 3 \), by-catch \( n = 2 \), lymphadenitis \( n = 1 \), heartworm related hemorrhage \( n = 1 \), or an undetermined cause \( n = 1 \).

**Statistics and models**

Statistical tests were performed using Excel 2000 (MS Office 2000) and \( R^4 \). All \( P \) values used were two-sided, and \( P \leq 0.05 \) was considered significant.

Models were fitted in \( R \). The amount of variability in the response value explained by a model was calculated as the difference between the total deviance and the residual deviance, divided by the total deviance.

**Results**

**Diagnosis of morbillivirus infection**

Out of 369 seals, 232 (63%) tested positive for morbillivirus infection. The proportion of seals testing positive was 168/361 (47%) by RT-PCR, 45/83 (54%) by IHC, 30/82 (37%) by IgM serology, and 87/326 (27%) by IgG serology.

The remaining seals (137/369, 37%) tested negative by RT-PCR or other assays even though the gross lesions were usually consistent with PDV infection (data not shown). We had already minimized the negative effect of autolysis on assay results by largely excluding carcasses in a moderate or advanced state of autolysis from our study. Therefore, we hypothesized a negative effect of the process of freezing and thawing on assay results. However, analysis of our data did not support this hypothesis: significantly fewer cooled than frozen seals were positive in the lungs by RT-PCR (12/51 cooled seals positive vs. 150/310 frozen seals, \( X^2 = 9.958, df = 1, P = 0.002 \), and no significant effect of storage conditions was observed in the other tests (IHC in lung sections: 11/38 cooled seals positive vs. 16/45 frozen seals, \( X^2 = 0.164, df = 1, P = 0.685 \); IgM serology: 18/46 cooled seals positive vs. 12/36 frozen seals, \( X^2 = 0.096, df = 1, P = 0.757 \); IgG serology: 19/53 cooled seals positive vs. 68/273 frozen seals, \( X^2 = 2.185, df = 1, P = 0.139 \).
Quantitative analysis of the 2002 phocine distemper epidemic in the Netherlands

The types of tissue testing positive by RT-PCR differed among seals. Brain tissue was frequently positive by RT-PCR (Table 1) when lung tissue was negative, and vice versa (Table 2). Assuming that seals testing positive in brain tissue had neurologic signs, this indicates that a number of neurologic cases did not have detectable viral RNA in their lungs at the time of death. Additionally, although comparable proportions of IgM-positive (7/16) and IgM-negative (11/28) seals tested RT-PCR positive in one or more tissues ($X^2 = 0.250, \text{df} = 1, P = 0.617$), the tissue distribution among these two groups differed significantly: IgM-positive seals were generally RT-PCR positive in the lung, while IgM-negative seals were generally RT-PCR positive in the brain (Table 2; Fisher’s exact test, $P = 0.015$).

Table 1: Tissue distribution of morbilliviral RNA and morbilliviral antigen in phocine distemper virus-infected harbour seals stored either cooled or frozen.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Storage condition</th>
<th>Any tissue</th>
<th>Lung</th>
<th>Kidney</th>
<th>Urinary bladder</th>
<th>Brain</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n° of seals positive for morbillivirus in:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Cooled</td>
<td>15</td>
<td>9</td>
<td>4</td>
<td>8</td>
<td>9</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>Frozen</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>IHC</td>
<td>Cooled</td>
<td>21</td>
<td>11</td>
<td>4</td>
<td>0</td>
<td>2*</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Frozen</td>
<td>24</td>
<td>16</td>
<td>10</td>
<td>1</td>
<td>nt</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

* : in cerebrum  
nt : not tested

Morbilliviral antigen expression by IHC was most frequently detected in lung and liver (Table 1). The cell types expressing morbilliviral antigen were bronchial epithelial cells (Figure 1), alveolar epithelial cells, alveolar macrophages, and syncytia in lung sections, bile duct epithelial cells in liver sections, mononuclear cells in spleen sections, pelvic epithelial cells in kidney sections, neurons and mononuclear cells in cerebrum sections, and transitional epithelial cells in urinary bladder sections. The tissue distribution of morbilliviral antigen expression by IHC often differed from that of viral RNA detected by RT-PCR (Table 2).

Seals that had IgG to morbillivirus but were negative by IgM, RT-PCR, and IHC may in principle have been infected before the 2002 PDV epidemic. Because PDV occurs as an epizootic disease in harbour seals in the North Sea and the only recorded occurrence of PDV prior to 2002 in this population was in 1988, seals that were exclusively IgG-positive had to be older than 13 years to have been infected with PDV in 1988 rather than in 2002. For IgG-positive seals that were 13 years or younger, we assumed that they had been infected in 2002 even though we did not have paired serum to prove seroconversion. In total, age was determined in 190/232 PDV cases (82%), of which 2% (3/190) were older than 13 years. Only one of these, a 16-year-old female, was positive by IgG and negative by the other three tests, and could thus have been infected in 1988.
The proportion of PDV cases with IgG to morbillivirus increased significantly with age (3 levels: <1-year-old, 1- or 2-year-old, ≥3-year-old; Fisher's exact test, n = 173, P = 0.003). The proportion of PDV cases with RT-PCR-positive lung tissue did not vary significantly with age, though it tended to decrease (Fisher's exact test, n = 173, P = 0.218). The relation of IgM and IHC results to age could not be determined in this sample because too few seals were tested by these methods.

Table 2: Comparison of diagnostic methods for morbillivirus infection in harbour seals infected with phocine distemper virus.

<table>
<thead>
<tr>
<th>Seal Nos</th>
<th>RT-PCR</th>
<th>Immunohistochemistry</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Kidney</td>
<td>Urinary Bladder</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>5</td>
<td>+</td>
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<td>+</td>
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<td>6</td>
<td>+</td>
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<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : positive  
- : negative  
(*) : cerebrum  
nt : not tested

Main gross lesions
The main gross lesions were pneumonia and interstitial emphysema (Figure 2). Pneumonia was characterized by raised consolidated areas that were bright red to dark-purple red. Both severity and distribution of pneumonia were determined in 210 of the 232 PDV cases. Pneumonia occurred in 198/210 cases (94%). The pneumonia was severe (174/198, 88%) or moderate (24/198, 12%), never mild. The distribution of the pneumonia was restricted to the ventral parts of the lungs in 69/198 (35%) cases (cranioventral, n = 30; caudoventral, n = 5; both, n = 34) and was more widespread in 129/198 (65%) cases (multifocal, n = 6; extensive, n = 123). In 12/210
(6%) cases there was no pneumonia: lungs were atelectatic \((n = 1)\), edematous \((n = 2)\), congested \((n = 2)\), both edematous and congested \((n = 2)\), or had no lesions \((n = 5)\). The presence and distribution of emphysema were determined in 226/232 cases. The vast majority of PDV cases had emphysema \((195/226, 86\%)\). Emphysema was pulmonary in 85/195 \((44\%);\) Figure 2), mediastinal in 178/195 \((91\%);\) Figure 2), pericardial in 152/195 \((78\%);\) retro-peritoneal in 102/195 \((52\%);\) and subcutaneous in 132/195 \((68\%);\) Retro-pharyngeal emphysema was observed in 2 cases. The distribution of the emphysema was variable. The most frequent distribution \((46/226, 20\%);\) was a combination of mediastinal, pericardial, retroperitoneal, and subcutaneous emphysema. Emphysema was rarely limited to the lungs \((8/195, 4\%);\)

\[\text{Figure 1: Lung; harbour seal No. 17. Morbillivirus antigen in bronchial epithelial cells. Immunoperoxidase histochemistry and hematoxylin, 400X (colour representation on back-cover).} \]

\[\text{Figure 2: Thoracic cavity; harbour seal No. 23. Pulmonary and mediastinal emphysema; histologically, there was severe broncho-interstitial pneumonia, associated with combined phocine distemper virus and Streptococcus equi subsp. zooepidemicus infections.} \]

Emphysema occurred significantly more often in cases with pneumonia than in those without \((\text{Fisher’s exact test, } n = 210, P < 0.001);\) However, the extent of emphysema \((3 \text{ levels: absent, thoracic only, thoracic and extra-thoracic})\) did not vary significantly with the severity of pneumonia \((2 \text{ levels: moderate, severe}; \text{Fisher’s exact test, } n = 198, P = 0.07);\) or with the distribution of pneumonia \((2 \text{ levels: ventral only, throughout the lungs}; \chi^2 = 1.015, n = 198, df = 2, P = 0.603);\)

Age \((3 \text{ levels: } <1\text{-year-old, 1- or 2-year-old, } \geq 3\text{-year-old})\) had a significant effect on the extent of emphysema \((\text{Fisher’s exact test, } n = 178, P = 0.001);\) with extra-thoracic emphysema occurring only very rarely in \(<1\text{-year-olds} \) while being very common in older seals \((\text{Figure 3});\) Seal age also had a significant effect on the severity of the pneumonia \((3 \text{ levels: absent, moderate, severe}; \text{Fisher’s exact test, } n = 175, P = 0.023);\) and on the distribution of pneumonia \((3 \text{ levels: absent, ventral only, throughout the lungs}; \text{Fisher’s exact test, } n = 175, P = 0.038);\) In particular, when pneumonia occurred in \(<1\text{-year-olds}, \) it was always severe and mostly extensive \((\text{Figure 3});\)
Other gross lesions

There were 21/232 (9%) PDV cases with other respiratory tract or thoracic cavity lesions: suppurative bronchitis (12/232, 5%), pulmonary abscesses (3/232, 1%), hemopurulent pleuritis (1/232, <1%), fibrinous pleuritis (1/232, <1%), pulmonary infarct (1/232, <1%), pulmonary hematoma (1/232, <1%), and hydrothorax (2/232, 1%). Of these cases, 19/19 (100%) also had pneumonia, 17/21 (81%) also had emphysema, 12/19 (63%) were RT-PCR positive in the lungs, and 9/21 (43%) were IgG positive.

There were 16/232 (7%) cases that had other significant gross lesions in other organ systems. These were purulent cystitis (1/232, <1%), otitis externa (2/232, 1%), phlegmon and facilities (1/232, <1%), hepatic atrophy possibly caused by porto-caval shunt (1/232, <1%), intestinal volvulus (2/232, 1%), perforation of internal organs by corpus alienum (2/232, 1%). Lymphadenopathy occurred in 6/232 cases (3%), affecting multiple lymph nodes (n = 4) or single non-thoracic lymph nodes (n = 2). In addition there was a neonate that had starved. Of these cases, 8/14 (58%) had pneumonia, 6/14 (43%) had emphysema, 4/16 (25%) were RT-PCR positive in the lungs, and 12/15 (80%) were IgG positive. Compared with the remainder of the
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PDV cases, these cases had significantly less pneumonia and emphysema (Fisher’s exact test, \( P \leq 0.001 \)), were significantly less RT-PCR positive (Fisher’s exact test, \( P \leq 0.001 \)) and significantly more IgG positive (Fisher’s exact test, \( P = 0.002 \)).

**Microscopic lesions**

The 29 cooled seals examined histologically were six <1-year-olds, nine 1- or 2-year-olds, ten ≥3-year-olds, and four of undetermined age. Microscopically, pneumonia occurred in 25/29 (86%) of the cooled PDV cases, characterized as bronchopneumonia (15/25, 60%) or broncho-interstitial pneumonia (10/25, 40%). Bronchopneumonia was generally marked, subacute, diffuse, and suppurative (Figures 4 & 5). In 2/14 cases, there were distinct, round, 3- to 10-μm-diameter, intracytoplasmic and intranuclear inclusion bodies in bronchial epithelium (Figure 4); in 4/14 cases, there was pulmonary emphysema (alveolar, interlobular, subpleural, or combinations of these) (Figure 6). Bronco-interstitial pneumonia was generally moderate, chronic, multifocal, and histiocytic (Figure 7). Worms (probably *Parafilaroides gymnurus*) were observed in the alveoli and bronchiolar lumina of 12/29 (41%) seals, all 0- or 1-year-olds. Diffuse acute moderate to severe congestion of the lung was nearly always observed (28/29, 97%; Figures 5–7).

**Figure 4:** Lung; harbour seal No. 6. Seal with suppurative broncho pneumonia associated with combined phocine distemper virus and *Bordetella bronchiseptica* infections. There are many neutrophils in the lumen and fewer among the bronchial epithelial cells. Note the eosinophilic intracytoplasmic inclusion bodies, characteristic for morbillivirus infection, in several bronchial epithelial cells. HE, 400X.

**Figure 5:** Lung; harbour seal No. 3. Seal with suppurative bronchopneumonia associated with combined phocine distemper virus and *Bordetella bronchiseptica* infections. Neutrophils fill the alveolar lumina. HE, 400X.
The two above-mentioned cases with inclusion bodies in bronchial epithelium also had intracytoplasmic inclusion bodies in the urinary bladder epithelium, and in one case also in renal pelvic epithelium and bile duct epithelium. The latter case had a mild necrotizing cystitis, pyelitis, and cholangitis. Splenic lymphoid depletion occurred in 5/29 (17%) cases, all ≥ 3-year-olds, splenic lymphoid hyperplasia in one case (1/29, 3%), and extramedullary erythropoiesis in 1 case (1/29, 3%). One seal (1/29, 3%) had marked adrenocortical atrophy. Another (1/29, 3%) had multifocal adrenocortical necrosis and multifocal necrotizing hepatitis. No lesions were observed in cerebrum or cerebellum.

The lung sections of 52/131 frozen PDV cases were interpretable. High to very high numbers of neutrophil occurred in 29/52 (56%) of these, corresponding with moderate to marked suppurative bronchopneumonia. These neutrophils were located in bronchi and sub-bronchial glands, alveoli and sometimes in interlobular spaces.

Organ weights
The consolidated lungs frequently felt heavy. To test whether PDV infection had a significant effect on the weight of lungs or other organs, we fitted two simple models using all the organ weight data available (PDV cases and non-PDV cases together). The weights of the two units of paired organs were summed and considered as 1. Organ weight generally increases with body size (growth), so the first model we created regressed organ weight (response variable) over body length (predictor). The correlation between organ weight and body length was positive for all organs examined, and the amount of variation in organ weight explained by this model was 68% for heart, 61% for kidneys, 55% for both lungs and liver, 48% for adrenals, 40% for brain, and 17% for spleen. By analysis of variance (ANOVA), we then compared this first model to a
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second model in which both body length and having PDV or not (PDV, 2 levels: 0 = other cause of death, 1 = PDV-case) were the predictors of organ weight. Adding PDV did not significantly improve the prediction of brain, heart, liver and spleen weight. However, it did significantly improve the model for lung, adrenal, and kidney weight (lungs, $F = 16.791$, $P < 0.001$, 67% lung weight variability explained; adrenals, $F = 14.231$, $P < 0.001$, 62% adrenal weight variability explained; kidneys, $F = 7.561$, $P = 0.008$, 67% kidney weight variability explained). Both predictors in these second models were positively correlated with the organ weights, indicating that PDV significantly increased the weight of lungs and adrenals and to lesser extent that of kidneys (model details not shown). The plotted data showed that this effect of PDV is most manifest in larger and therefore older seals (Figure 8).

**Figure 8:** Weight of lungs, kidneys, adrenals, and brain of harbour seals infected with phocine distemper virus (closed diamonds) and non-infected seals (open squares) regressed over seal body length (cm) shows that the first three organs are heavier in infected seals, in particular the longer (older) seals.
We hypothesized that suppurative bronchopneumonia would result in heavier lungs. However, there was no association between moderate or high numbers of neutrophils and lung weight in PDV cases (the ratio lung weight over body length compared between 18 PDV cases with high numbers of neutrophils and 13 PDV cases with no or few neutrophils; two-sample t-test assuming equal variances, \( P = 0.964 \)).

**State of nutrition**

Stomach contents were recorded for 210 PDV cases. The majority had empty stomachs (195/210, 93%). The remaining 15 had one or more corpora aliena (9/210, 4%), fish bones (3/210, 1%), or fish (i.e., a recent meal, 3/210, 1%). The corpora aliena were pieces of plastic (\( n = 3 \)), wire booms (\( n = 2 \)), a spring boom and 1 French boom, both parts of pater noster rigs used in sea angling), a spring (\( n = 1 \)), an iron thread (\( n = 1 \)), foil (\( n = 1 \)), a string (\( n = 1 \)), stones (\( n = 2 \)), and a piece of wood (\( n = 1 \)). The three seals with fish in their stomach were 0, 3, and 16 years of age. The <1-year-old was positive by RT-PCR in the brain and bladder, the 3-year-old was positive by RT-PCR in the lungs (only tissue tested), and the 16-year-old was the PDV-case only positive for IgG as previously mentioned.

For comparison, the seven non-PDV cases whose deaths were associated with worm or bacterial infections, and one mesenteric torsion case, had empty stomachs. The two cases whose deaths were associated with corpora aliena and the other mesenteric torsion case had corpora aliena. The five cases whose deaths were associated with by-catch had fish in their stomachs. In the nine cases for which the cause of death was undetermined, any of these three stomach content situations was found.

The average blubber thickness of PDV cases (\( n = 231 \)) was 13.5 mm (range 0 to 26 mm). It differed significantly from that of the non-PDV cases whose deaths were associated with by-catch (31.8 mm, range 15 to 45 mm; \( n = 5 \); Mann-Whitney test, \( P = 0.001 \)), and seemed more comparable with those of non-PDV cases that died from other causes than by-catch.

Blubber thickness increases with age and changes seasonally with molt and reproductive activities\(^{22,49,63}\). Therefore, we regressed blubber thickness (mm) over age (years) and then over date of stranding, the latter also for different sex-age classes (female <1-year-olds, female 1- to 2-year-olds, ≥ female 3-year-olds; male <1-year-olds, male 1- to 2-year-olds, male ≥ 3-year-olds). There was a significant trend for blubber thickness to decrease with age (\( r^2 = 0.039, n = 189, P = 0.007 \)). Blubber thickness also decreased significantly over time during the epidemic (\( r^2 = 0.089, n = 189, P \leq 0.001 \)) among <1-year-olds (female, \( y = -0.174x + 61, r^2 = 0.429, n = 13, P = 0.015 \); male, \( y = -0.145x + 50, r^2 = 0.445, n = 15, P = 0.007 \)) and seals ≥ 3-year-olds (female, \( y = -0.141x + 46, r^2 = 0.299, n = 35, P = 0.001 \); male, \( y = -0.141x + 46, r^2 = 0.185, n = 49, P = 0.002 \)). There was no effect of date of stranding on blubber thickness of 1- or 2-year-olds (females, \( r^2 = 0.004, n = 47, P = 0.689 \); males, \( r^2 = 0.003, n = 30, P = 0.95 \)).
**Phocine herpes virus 1 coinfection**

Nine of 169 (5%) PDV cases tested were positive by PCR for phocine herpes virus 1 infection. There were five females (a 3- and a 4-year-old, two 1-year-olds, and two 100-cm-long female) and four males (a 5-year-old, two 4-year-olds, and a 3-year-old).

**Bacterial coinfections**

Fourteen bacterial genera or species were cultured from the PDV cases examined (Table 3, Figure 9). *B. bronchiseptica* was the most frequently isolated bacterium (24/36, 66%). It was always cultured from lung tissue (24/24) and sometimes additionally from other tissues examined (15/24; Table 3). The seals with *B. bronchiseptica* infection were <1-year-old (*n* = 5), 1-year-old (*n* = 5), ≥3 years or older (*n* = 6), or of undetermined age (*n* = 7). In 8/36 (22%) cases, *B. bronchiseptica* was cultured from the lungs (and other organs) in association with high numbers of other bacteria; in 4/36 (11%) cases, it was the only specific bacterium cultured from the lungs; and in 12/36 (33%) cases, it was the only specific bacterium cultured from all organs examined.

**Table 3:** Tissue distribution of bacteria isolated from harbour seals infected with phocine distemper virus.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Any tissue (n=36)</th>
<th>Lung (n=36)</th>
<th>Trbrln(*) (n=36)</th>
<th>Liver (n=36)</th>
<th>Spleen (n=36)</th>
<th>Kidney (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td>24</td>
<td>24 (++++)</td>
<td>12 (++++)</td>
<td>2 (++)</td>
<td>3 (++)</td>
<td>2 (±)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> &amp; other coliforms</td>
<td>8</td>
<td>6 (++++)</td>
<td>2 (++++)</td>
<td>3 (++++)</td>
<td>1 (++)</td>
<td>1 (++)</td>
</tr>
<tr>
<td><em>Streptococcus sp.</em></td>
<td>6</td>
<td>5 (++++)</td>
<td>1 (++++)</td>
<td>1 (++)</td>
<td>3 (±)</td>
<td>2 (++)</td>
</tr>
<tr>
<td><em>Gemella sp.</em></td>
<td>5</td>
<td>5 (++++)</td>
<td>2 (++)</td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
</tr>
<tr>
<td><em>Clostridium sp.</em></td>
<td>5</td>
<td>3 (++++)</td>
<td>2 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>3</td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
</tr>
<tr>
<td><em>Proteus sp.</em></td>
<td>2</td>
<td>2 (ND)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>1</td>
<td>1 (++)</td>
<td></td>
<td></td>
<td></td>
<td>1 (±)</td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td>1</td>
<td>1 (++)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phytophthora davidsonii</em></td>
<td>1</td>
<td>1 (++)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>1</td>
<td>1 (++)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Psychrobacter phenylpyruvici</em></td>
<td>1</td>
<td>1 (±)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1</td>
<td>1 (++)</td>
<td></td>
<td>1 (±)</td>
<td>1 (±)</td>
<td>1 (±)</td>
</tr>
<tr>
<td><em>Vibrio sp.</em></td>
<td>1</td>
<td>1 (±)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*) Trbrln: tracheo-bronchial lymph node
(‡): Potential postmortem contaminant.
Numbers cultured (in parentheses): ± = occasional; + = few; ++ = moderate; +++ = many; ++++ = very many; ND = not determined.
In the remaining 12/36 (34%) cases, B. bronchiseptica was absent. In these cases, other specific bacteria were isolated in significant numbers in lungs and other organs (5/36, 14%, including a case of Staphylococcus aureus septicemia), in small or undetermined numbers in one or few organs (5/12, 14%), or were absent (2/36 unspecified mixed cultures, 6%).

Six PDV cases with other gross lesions were among those from which bacteria were cultured: two bronchopneumonia cases (B. bronchiseptica with Streptococcus equisimilis or with Clostridium perfringens, from respiratory tract); a pulmonary abscess case (S. equi subsp. zooepidemicus, from multiple organs); a pulmonary hematoma case (B. bronchiseptica with S. zooepidemicus, both from multiple organs); a generalized lymphadenopathy case (Gemella sp., from lung and multiple lymph nodes), and the hepatic atrophy case (Escherichia coli, from lung, tracheo-bronchial lymph node and liver; Enterobacter sp. from lungs, Aeromonas sp. from kidney).

When B. bronchiseptica was the only specific bacterium isolated from the tissues examined, there was a straightforward relationship with the number of neutrophils in lung sections: there were no or few neutrophils when B. bronchiseptica was isolated from lung tissue only; there were moderate to large numbers of neutrophils when B. bronchiseptica was isolated from the lungs and other tissues. However when other bacteria were isolated in moderate to large numbers from the lungs, with or without B. bronchiseptica, no consistent pattern was detected with regard to neutrophil numbers.

The species of bacteria isolated from the PDV cases in 2002 only partially overlapped with those isolated from non-PDV cases in 2000–2001 (Figure 9). Notably, B. bronchiseptica was not isolated
from the non-PDV cases. In contrast, Corynebacterium sp. was not isolated from the PDV cases, whereas it was isolated from 3/8 non-PDV cases where it was twice associated with cause of death. Brucella sp. was not isolated.

**Parasitic coinfections**

Macroscopically, lungworms (identified as Otostrongylus circumlitus and Parafilaroides gymnurus) were observed in 25/226 (11%) of the PDV cases, heartworms (identified as Acanthotheiloneema spirocauda) in 42/221 (19%), and stomach worms (identified as Pseudoterranova decipiens and Contracaecum osculatum) in 150/227 (66%). The frequency distributions of the parasites were highly aggregated as expected for macroparasitic infections (Figure 10). The distributions of lung- and heartworms were quite comparable, and different from that of stomach worms (Figure 10).

![Figure 10](image)

The frequency distributions of worm burdens in harbour seals infected with phocine distemper virus (lungworms, closed diamonds, n of seals = 226; heartworms open squares, n of seals = 221; stomach worms, open triangles, n of seals = 227).

All three worm burdens and seal age were determined for 179/232 seals. Lungworms were observed in 21 of these, heartworms in 28 and stomach worms in 150. The first <1-year-old in this sample stranded on 14 August 2002, the first <1-year-old with lungworms on 29 August, with heartworms on 27 August, and with stomach worms on 8 September 2002. Lungworms were observed most frequently and with the highest burdens in <1-year-olds, and heartworms most frequently and with the highest burdens in 1- and 2-year-olds (Figure 11). Stomach worms were seen in all age categories, but the highest burdens occurred in seals ≥3 years old (Figure 11). Stomach worm burden (4 levels: no worms; 1–10 worms; 11–100 worms; >100 worms) increased significantly with age in males but not in females (males, $r^2 = 0.172$, $n = 87$, $P < 0.001$; females, $r^2 = 0.007$, $n = 92$, $P = 0.420$).

The presence and number of lice were reported in 212/232 cases. Lice were present in 29/212 (14%) of the cases (16 cases with 1–10 lice, 10 with 11–100 lice, and three with 100 lice). Lice were most frequent and with the highest burdens in 1- and 2-year-olds. However, lice can drop off carcasses once the seal is dead, and therefore this may be an underestimation of the number of seals with lice as well as of louse numbers.
Correlating pathology findings and related laboratory diagnostic analyses in PDV cases and comparing these with those of non-PDV cases have provided further insight into the pathology of PDV infection in free-living harbour seals. Because our study was limited to seals found dead or moribund, these findings are not necessarily representative of the disease process as it may occur within the overall population. Also, although levels of contaminants occurring in seals may be immunosuppressive and therefore affect the disease process, we did not investigate this during the current study16. We sequentially discuss a number of points raised by the PDV assay results, the lesions, and lastly the coinfections.

Analysis of the PDV assay results showed differential temporal presence of virus in lung and brain, and age-related differences in disease course. First, the results suggested differential periods of presence of virus in lungs and brains. Clearance of virus from host tissues occurs in morbillivirus infections, but clearance from the brain is not efficient in canine distemper cases with brain involvement in dogs1,20,42,47. Such cases also tend to have a longer disease course than average1. In this study, seals positive by RT-PCR in the brains tended not to be positive by RT-PCR.
in the lungs, which would fit with an efficient virus clearance from most tissues including the lungs, but not from the brains. These results indicate that both lung tissue and brain tissue need to be tested by RT-PCR when PDV is suspected.

Second, associating PDV assay results to seal age provided support for age-related differences in disease course. Indeed, the proportion of seals with IgG antibodies increased significantly with age. There was also an indication for such a trend for IgG in seals infected during the 1988 epidemic, and evidence for such a trend for neutralizing antibodies. For the latter, 4 possible explanations were discussed. The first was that juvenile seals were less exposed to the virus; this is inconsistent with the PCR-results in the present study. The second was that older seals would have more anamnestic responses; this is inconsistent with the present study because only a few dead adult seals were old enough to have been alive during the 1988 epidemic. The last two explanations were greater mortality among young seals and age-related compromised immune responses in young seals; these are not inconsistent with the results of our study. A variant of the latter is a lengthier disease course in older seals.

Analysis of PDV-associated lesions showed influences of the seal’s anatomy and physiology on the development of emphysema, an effect of age on disease course, evidence of dehydration, an effect of age on blubber thickness, and different disease characteristics in cases with severe gross nonrespiratory tract lesions. First, emphysema is likely to develop as a result of inflammation and airway obstruction, a concept supported in this study by the positive correlation between the occurrence of pneumonia and emphysema in the PDV cases. Pneumonia is a frequent feature of morbillivirus infections in all host species. However, to our knowledge, emphysema has been associated with morbillivirus infection in seals and cattle but not in dogs. A possible explanation is that seals and cattle—but not dogs—have well-developed interlobular septa that prevent collateral ventilation in case of airway obstruction, resulting in interstitial emphysema.

Second, linking pneumonia and emphysema to seal age suggested age-related differences in the development of lesions. In particular, the extent of emphysema varied significantly with age class rather than with severity and distribution of pneumonia: most <1-year-olds had severe and extensive pneumonia, but rarely had extra-thoracic emphysema. In contrast, extra-thoracic emphysema in older seals was common regardless of the extent and severity of the pneumonia. A possible explanation for this difference is a lengthier disease course in older seals. This may be analogous to cattle with protracted interstitial pneumonia, where emphysema also spreads from the lungs to the mediastinum and the skin of the back. Another explanation could be that physiological diving responses and oxygen storage capacity are not fully developed in <1-year-olds, so <1-year-olds perform shorter dives than older seals. Normally pinnipeds exhale before diving, and the air that remains in the alveoli is squeezed out and into the bronchi and trachea as pressure causes the lungs collapse. If the airways are obstructed, as in seals with
PDV infection, air in the alveoli might rupture the alveolar walls during long dives, resulting in emphysema.

Third, the effect of PDV on nutritional state and organ weights suggests a role for dehydration. The empty stomachs of PDV cases indicate that feeding and associated water intake from prey was impeded. Further evidence for anorexia was that blubber reserves of PDV cases were on average reduced compared to those of by-caught seals (this study) or culled seals. Presumably PDV cases have higher water requirements than normal, because they excrete large amounts of fluid via oro-nasal and lacrimal secretions and diarrhea. Therefore, it is likely difficult for these seals to maintain their water and electrolyte balances. The increased kidney and adrenal weights that we observed in PDV cases fit with dehydration. Water-deprived rodents also show significantly increased renal and adrenal weights, the first possibly caused by the hypertrophy of the renal medulla. Future studies of stranded seals might address the question of dehydration through measurement of urea nitrogen in vitreous humor fluid.

Fourth, we next found that blubber thickness of the PDV-infected seals decreased with age, and with date of stranding in seals ≥3 years old and in <1-year-olds. In seals ≥ 3 years old, these results suggest either lengthier disease or coincidence with physiological fat loss (e.g., because of reproductive activities), or a combination of these. In <1-year-olds, post weaning fat loss seems a more plausible explanation for the observed time trend than an effect of maternal antibodies on disease duration because antibodies to PDV were virtually absent in the population.

Fifth, PDV cases with macroscopic evidence of significant nonrespiratory tract lesions were significantly more RT-PCR negative and IgG positive than the other PDV cases, and fewer had pneumonia and emphysema. These findings suggest these PDV cases had a different and lengthier disease course than most PDV cases.

Analysis of coinfections showed that bacterial pneumonia occurred frequently and provided evidence for an immunosuppressive effect of PDV. First, suppurative pneumonia indicative of bacterial coinfection was commonly diagnosed by histologic analysis of lung tissues. This was surprising, because macroscopic evidence of suppurative pneumonia was rare. A possible explanation for this discrepancy might be that a high proportion of the bacterial infections in the lung were peracute to acute and had not yet led to macroscopically visible changes.

Second, the indications for immunosuppression were the frequent presence of *B. bronchiseptica* in the lungs, and the occurrence of lungworm infections in older seals. *B. bronchiseptica* is often isolated from lungs of seals and other carnivores during PDV or canine distemper virus (CDV) epidemics, but not outside these epidemics. In dogs *B. bronchiseptica* is often chronically present in the upper respiratory tract, infection occurring early in life and followed by a certain resistance to severe disease. It is thought that when hosts are immunosuppressed, the
bacterium can invade and colonize the lower respiratory tract and cause bronchopneumonia. The same principle applies for lungworms, as these tend to infect and cause disease in harbour seals early in life, after which surviving hosts usually have a certain degree of immunity to reinfection and disease.  

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We are grateful to the people who helped with the collection and necropsy of the seal carcasses stranding along the Dutch coast. We also thank Barry Ziola, Department of Microbiology and Immunology, Saskatoon, Saskatchewan, Canada, for providing the rabbit serum for the detection of morbillivirus antigen by immunohistochemistry, Herman Cremers for identifying macroparasites, and Frank van der Panne for his assistance with Figures 1, 2, and 4–7.
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Toxicology
No sustained decline of PCB and DDT burdens in Dutch harbour seals (*Phoca vitulina*)

Submitted

Natsuko Kajiwara • Jolianne M. Rijks • Thijs Kuiken • Karst van der Meulen • Tomoyuki Tonegi
Yoko Ochi • Masao Amano • Shinsuke Tanabe • Albert D.M.E. Osterhaus

Center for Marine Environmental Studies, Ehime University, Matsuyama, Ehime, Japan; Department of Virology, Erasmus MC, Rotterdam, The Netherlands; Seal Rehabilitation and Research Center, Pieterburen, The Netherlands; Dutch Wildlife Health Centre, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; Research Center for Material Cycles and Waste Management, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan; Department of Animal Sciences, Teikyo University of Science & Technology, Yatsusawa, Uenohara, Yamanashi, Japan.
Since the 1970s, policies have been implemented to decrease the burden of harmful anthropogenic contaminants in the environment. This initially led to downward trends of major organohalogens in sediment and biota of the North Sea. More recent data on trends of organohalogen levels in harbour seals (Phoca vitulina) as indicators of North Sea ecosystem health are largely lacking. Juvenile harbour seals stranded along the Dutch coast during the phocine distemper virus (PDV) epidemic in 2002 (n=18) and throughout the preceding decade (n=27) were analysed for polychlorinated biphenyls (PCBs), organochlorine pesticides and polybrominated diphenyl ethers (PBDEs). Hexachlorocyclohexane isomers (HCHs) and hexachlorobenzene (HCB) levels showed a significant decline, but all remaining compounds, including PCBs, dichlorodiphenyltrichloroethane and its metabolites (DDTs) and PBDEs, did not. PCBs remained at levels previously associated with immune suppression. These results indicate that, despite reduced input, certain major organohalogens have stabilized in harbour seal tissues at potentially harmful levels.

Harbour seals are at risk of suffering ill health from anthropogenic contaminants such as organohalogens. Like human beings, they are top predators in the North Sea ecosystem and belong to the highest trophic levels of the food chain. As a result, chemically stable organohalogens tend to accumulate more in their tissues than in those of species at lower trophic levels. In the late 1970s and early 1980s, high levels of organohalogens, particularly polychlorinated biphenyls (PCBs), were associated with poor reproductive performance in harbour seals. Furthermore, when a phocine distemper virus epidemic decimated the North Sea harbour seal population in 1988, it was speculated that pollution-related immune suppression had contributed to the severity and extent of the epidemic. It was subsequently shown that the levels of PCBs and dichloro-diphenyl-trichloroethane and its metabolites (DDTs) in tissues of North Sea harbour seals from that period exceeded those associated with immune suppression.

When a second PDV epidemic struck the North Sea harbour seal population in 2002 and caused a similar level of mortality as the previous epidemic 14 years earlier, it was again speculated that pollution had played a role. However, recent data on organohalogen levels in harbour seal tissues from this region are largely lacking. This prompted us evaluate trends for levels of organochlorine compounds, namely PCBs, DDTs, tris(4-chlorophenyl)methanol (TCPMOH), chlordane related compounds (CHLs), tris(4-chlorophenyl)methane (TCPMe), heptachlor epoxide (HP-epon), hexachlorocyclohexane isomers (HCHs) and hexachlorobenzene (HCB), as well as for the newly introduced flame retardant polybrominated diphenyl ethers (PBDEs) in harbour seal blubber. To this end, blubber samples from 45 juvenile harbour seals that had stranded along the Dutch coast during the 2002 epidemic (n = 18) and in the preceding decade (n = 27) were analysed for levels of these compounds (Materials and Methods in Annex). Blubber samples from juvenile seals were chosen for this study not only because they can readily be obtained for
monitoring, but also to limit effects of sex and duration of environmental exposure.

Organochlorines and PBDEs were detected in all the blubber samples analysed (Table 1). On a lipid weight basis, the highest mean values of organochlorines were found for PCBs (mean, 28 μg/g; median, 16 μg/g; range, 2.6-270 μg/g), DDTs (mean, 3.0 μg/g; median, 1.8 μg/g; range, 0.18-20 μg/g), and TCPMOH (mean, 0.80 μg/g; median, 0.50 μg/g; range, 0.040-7.7 μg/g) (Figure 1, left panel). This was followed in decreasing order by CHLs (mean, 290 ng/g; median, 200 ng/g; range, 26-1900 ng/g), HP-epox (mean, 61 ng/g; median, 47 ng/g; range, 10-340 ng/g), TCPMe (mean, 32 ng/g; median, 20 ng/g; range, 3.0-260 ng/g), HCHs (mean, 18 ng/g; median, 14 ng/g; range, 6.0-66 ng/g), and HCB (mean, 5.0 ng/g; median, 3.6 ng/g; range, 1.0-38 ng/g) (Figure 1, right panel). Levels of PBDEs were on average 380 ng/g on a lipid weight basis (median, 180 ng/g; range, 37-2500 ng/g) with predominant contribution by BDE-47 (Figure 1, right panel). None of the samples analysed contained detectable amounts of BDE-209 (Table 1).

![Figure 1: Average levels of organohalogen compounds in the blubber of 45 juvenile harbour seals stranded along the coast of the Netherlands in the period 1993-2002.](image)

The organochlorines, including PCBs and DDTs, and PBDEs did not show a significant downward trend in tissue levels during the period 1993 to 2002, based on multiple regression analysis (in Annex). As the only exceptions, there were significant downward trends in the tissue levels of organochlorine pesticides HCHs (13.3% per year, \(p = 8 \times 10^{-7}\)) and HCB (12.9% per year, \(p = 1 \times 10^{-5}\)) (Table 2). In the two models, for HCHs and HCB, in which “year” was retained as predictor, it explained 36% (HCHs) and 35% (HCB) of the variation in contaminant levels between individuals. As expected, there was a significant inverse correlation between the levels of each of the compounds and blubber thickness (p-values ranging from \(7 \times 10^{-4}\) to 0.02), with exception of HCB (p=0.75). The average reduction per mm blubber thickness was 2.5% for HCH and 4.3 to 6.3% for all other correlating compounds. Body length had no significant effect on the levels of organohalogens observed, although female sex significantly increased the levels of TCPMOH by 92% (p=0.03) and TCPMe by 88% (p=0.02). The interaction term between sex and body length did not improve the models. Overall, the models explained between 13% (PBDEs) and 42% (HCHs) of the variation in contaminant levels between individuals (Figure 2; Table 2). Combining the data...
from our study with toxicological studies of juvenile harbor seals from the southern North Sea in the 1970s and 1988 allowed us to analyze the trends in levels of PCBs, DDTs, and HCHs in period 1974 to 2002 (Figure 3). Although an effect of location cannot be excluded, this analysis suggests that, after a general decline in levels between the 1970s and 1988, the trends of these three organochlorines have diverged. The levels of HCHs have continued to decline. In contrast, the levels of PCBs and, to a lesser degree, DDTs in harbor seal tissues have leveled off in the last 14 years. Although the relative contribution of metabolized persistent derivates (p,p'-DDE) of DDT tended to increase, this trend was not significant (p = 0.07). We therefore cannot conclude that DDT release into the environment was reduced significantly in the decade studied.

Figure 2: Organochlorine levels in the blubber of 45 juvenile harbour seals stranded along the coast of the Netherlands in the period 1993-2002 over year of stranding or blubber thickness. Lines indicate significant trends.
Table 1: Biological data and organohalogen concentrations in the blubber of 45 juvenile harbour seals stranded along the Dutch coast in the period 1993 to 2002. The last 18 seals in the table died during the 2002 phocine distemper virus epidemic.

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**α-g Lipid weight**: PCBs, 7PCBs, DDTs, TCPMOH

**ng/g Lipid weight**: CHLs, HP-epox, TCPMe, HCHs, HCB, PBDEs

**Abbreviations**
- **BL**: body length
- **BT**: blubber thickness
- **PCBs**: polychlorinated biphenyls
- **7PCBs**: CB28+CB52+CB101+CB(118+149)+CB138+CB153+CB180
- **DDTs**: dichlorodiphenyltrichloroethane and its metabolites
- **CHLs**: chlordane-related compounds
- **HCHs**: hexachlorocyclohexane isomers
- **HCB**: hexachlorobenzene
- **HP-epox**: heptachlor epoxide
- **TCPMe**: tris(4-chlorophenyl)methane
- **TCPMOH**: tris(4-chlorophenyl)methanol

**Note**: No sustained decline of PCB and DDT burdens in Dutch harbour seals.
**Table 2:** Results of multiple regression analyses on log10-transformed lipid weight values of each of the organohalogen contaminants in blubber as response value, with year as predictor and blubber thickness as confounder.

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<th>Coefficient</th>
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<td>15%</td>
<td>BT (mm)</td>
<td>&lt;0.01</td>
<td>-0.027</td>
<td>0.009</td>
<td>-6.0%</td>
<td></td>
<td>[-9.8%; -2.1%]</td>
</tr>
<tr>
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<td>BT (mm)</td>
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<td>0.011</td>
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<td></td>
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<tr>
<td></td>
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<td>0.025</td>
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<td></td>
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<td></td>
<td>[-7.5%; -2.3%]</td>
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<td>0.004</td>
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<td>[-4.3%; -0.7%]</td>
</tr>
<tr>
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</tr>
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<td>0.010</td>
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<td></td>
<td>[-9.2%; -0.5%]</td>
</tr>
<tr>
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<td></td>
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<td>-0.040</td>
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na: not applicable
No sustained decline of PCB and DDT burdens in Dutch harbour seals (*Phoca vitulina*)

**Figure 3:** Trend of $\log_{10}$ transformed mean values of three organochlorine compounds in the blubber of juvenile harbour seals using different studies. Minimum sample size was set at 3. Juvenile seals were selected based on maximum age of 2 years and maximum length of 105 cm. Triangles represent seals stranded in the Netherlands (Wadden Sea and North Sea), plusses in Germany (Wadden Sea), diamonds in Denmark (Wadden Sea and Limfjord), and squares in the U.K. (The Wash and Norfolk). Grey symbols represent transformed values. Prior to log-transformation, values were multiplied by 0.85 for conversion from lipid weight to wet weight, and by 0.65 for conversion from total PCB to 7 ICES PCB.
The third analysis we performed was to compare mean tissue levels of PCBs, DDTs, and HCB in juvenile harbour seals that died in 2002 just before or during the PDV epidemic (n = 23) with those found at the end of a semi-field feeding experiment carried out with juvenile harbour seals in the early 1990s. In that experiment, several negative effects on immune function were observed in seals that were fed herring from the heavily polluted Baltic Sea compared to seals that were fed herring from the less contaminated Atlantic Ocean. These effects could be attributed primarily to PCBs, even though other immunotoxic contaminants were present. Comparing the contaminant levels in seals from 2002 and those fed on Baltic Sea herring, there were no significant differences in mean levels of PCBs (p = 0.15), while mean levels of DDTs and HCB in 2002 seals were significantly lower than those in the Baltic Sea group (p < 0.01). These data suggest that PCBs in harbour seals that died during the 2002 PDV epidemic occurred at levels comparable to those associated with immunosuppression in the semi-field experiment.

The main origins of organohalogen pollution are industrial and agricultural sources that pollute the marine environment via riverine and other land or sea based discharges, as well as atmospheric fall-out. Published data on trends in organohalogen residues in the sediment and biota in the North Sea area are limited and fragmentary. PCB levels in sediment declined significantly in the period 1981 to 1996 as a result of specific measures taken from the 1970s onwards in the industrialized world. However, this decline has levelled off in recent years (1991-2001). Also, no declining trend could be measured in mussels and fish in the late 1980s. DDT levels in sediment and fish from the North Sea coastal environment also have decreased following discontinuation of its production and use in the industrialized world in the early 1970s. However, the use of DDT has long been continued in tropical areas, albeit at a smaller scale than before. Our observation that the ratio of metabolized p,p'-DDE over total DDTs tended to increase, although not statistically significant, is in line with studies on sediment and fish, suggesting that exposure of the North Sea coastal environment to DDT is decreasing. HCB levels in sediment and HCH and HCB levels in several fish species from the North Sea have declined after their use was largely discontinued from the late 1980's onward. However, \(\gamma\)-HCH (lindane), which is the only insecticidal isomer of HCH, is still in limited use, and HCB is still produced unintentionally, under strict regulations, as a by-product of chlorinated organic solvents.

For the remaining compounds (TCPMOH, TCPMe, CHLs, HP-epox, and PBDEs), there was no evidence for a trend in the decade studied, and historical data for marine mammals are too limited for comparison. It is noteworthy that levels of PBDE flame retardants did not increase, which may reflect the timely implementation of restrictions on the use of the most bioaccumulating compounds.

Collectively, the results of this study show that, except for HCB and HCHs, the levels of major organohalogen contaminants, including PCBs and DDTs, have not declined in tissues of...
No sustained decline of PCB and DDT burdens in Dutch harbour seals (*Phoca vitulina*)

harbour seals in the last decade, and still exceed the levels at which immunosuppressive effects have been measured. Therefore, these contaminants may have contributed to the severity and extent of the PDV outbreak that caused approximately 50% mortality in the North Sea harbour seal population in 2002\textsuperscript{12}. It should be realised that only an estimated one-third of the total PCB burden produced had been released into the environment by 1988\textsuperscript{27}. Taking into account the large still existing stocks, the importance of the atmospheric route of input into the oceans, and the extreme biomagnification\textsuperscript{3}, we need to take into account, both for the health of humans and wildlife, that PCB levels will continue to persist in the environment in the near future.

**Acknowledgements**

We thank Rene Eijkemans for kindly providing advice for the statistical analysis. This study was supported by “21st Century COE Program” and “Global COE Program” from the Ministry of Education, Culture, sports, Science and Technology, Japan, Japan Society for the Promotion of Science, and Dutch Ministry of Agriculture, Nature and Food Quality.
Reference List


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Annex

Materials and Methods

Samples
Harbour seals (*Phoca vitulina*) used in this study were found stranded on the coasts of The Netherlands between 1993 and 2002. Sampling locations and biological data of 45 cases analyzed in the present study are given in Table S1. Blubber samples were excised from all the dead animals, put in polyethylene bags, and kept in a deep freezer at -20°C of the Environmental Specimen Bank for Global Monitoring (es-BANK) at Ehime University (S1) until chemical analysis.

To limit the sample to juvenile seals (2 years or less), seals of 105 cm or less body length (nose to tail tip) were selected. In addition, for the 18 seals from the PDV epidemic age was confirmed using a single canine tooth removed from the lower jaw. Longitudinal, decalcified and haematoxylin-stained thin sections (20 µm) of each tooth were prepared following the procedure described by Kasuya (S2). For each tooth, all the growth layer groups (GLG) were counted in both dentine and cementum, three times independently, and the median value was taken as the GLGs. The annual accumulation of a dentinal and cemental GLG is widely accepted for pinniped species (e.g., S3, S4) and therefore one GLG was assumed to correspond 1 year.

Chemical Analyses
Organochlorine compounds including PCBs, DDTs (dichlorodiphenyltrichloroethane and its metabolites), HCHs (hexachlorocylohexane isomers), CHLs (chlordan related compounds), HCB (hexachlorobenzene), HP-eopx (heptachlor epoxide), TCPMe [tris (4-chlorophenyl) methane] and TCPMOH [tris (4-chlorophenyl) methanol] were analyzed following the methods described by Kajiwara and others (S5). Briefly, approximately 2 g of blubber were homogenized with anhydrous sodium sulfate and extracted in a Soxhlet apparatus for 7-8 hrs using a mixture of diethyl ether and hexane. An aliquot of the extract was subjected to a gel permeation chromatography column (GPC; Bio-Beads S-X3, Bio-Rad Laboratories, CA, 2 cm id. and 50 cm length) for lipid removal. The GPC fraction containing organochlorines was concentrated and passed through an activated Florisil column for clean up and fractionation. Quantification of PCBs and most of organochlorine pesticides was performed using a GC (Agilent 6980N) equipped with a micro-electron capture detector (micro-ECD) and an auto-injection system (Agilent 7683 Series Injector). The GC column used for organochlorines analysis was a fused silica capillary (DB-1, 30 m x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific Inc.). Identification and quantification of HP-eopx, TCPMe and TCPMOH were performed using a GC-MSD (Agilent 5973N) in selective ion monitoring (SIM) mode equipped with an auto-injection system (Agilent 7683 Series Injector). The concentration of individual organochlorines was quantified from the peak area of the sample to that of the corresponding external standard. The PCB standard used for quantification was a mixture of sixty-two PCB isomers and congeners (BP-MS) obtained from Wellington Laboratories Inc., Ontario, Canada. Concentrations of individually resolved peaks of
PCB isomers and congeners were summed to obtain total PCB concentrations.

Analysis of PBDEs was performed following the procedure described by Ueno and others (S6) with slight modification. Another aliquot of the extract, after adding 5 ng of internal standards (13C12-labeled BDE-3, BDE-15, BDE-28, BDE-47, BDE-99, BDE-153, BDE-154, BDE-183 and BDE-209), was added to a GPC column for lipid removal. The GPC fraction containing organohalogen was concentrated and passed through 1.5 g of activated silica gel S-1 (Wako Pure Chemical Industries Ltd., Japan) column with 5% dichloromethane in hexane for clean up. 13C12-labeled BDE-139 was added to final solution prior to GC-MSD analysis. Quantification was performed using a GC (Agilent 6890N) equipped with MSD (Agilent 5973N) for mono- to hepta-BDEs, and GC (Agilent 6890N) coupled with MSD (JEOL GCmate II) for deca-BDE, having an electron impact with SIM mode. GC columns used for quantification were DB-1 fused silica capillary (J&W Scientific Inc.) having 30 m x 0.25 mm i.d. x 0.25 µm film thickness for mono- to hepta-BDEs, and 15 m x 0.25 mm i.d. x 0.1 µm film thickness for deca-BDE. Ten major congeners of PBDEs (BDE-3, BDE-15, BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209) were quantified in this study. All the congeners were quantified using the isotope dilution method to the corresponding 13C12-labeled congener. Recovery of 13C12-labeled BDE congeners added as recovery surrogates prior to sample clean-up ranged between 60-120%.

Procedural blanks were analyzed simultaneously with every batch of five samples to check for interferences or contamination from solvents and glassware. Lipid contents were determined by measuring the total non-volatile solvent extractable material on sub-samples taken from the original extracts. The concentrations of organohalogen are expressed on a lipid weight basis unless otherwise specified.

For quality assurance and control, our laboratory participated in the Intercomparison Exercise for Persistent Organochlorine Contaminants in Marine Mammals Blubber, organized by the National Institute of Standards and Technology (Gaithersburg, MD) and the Marine Mammal Health and Stranding Response Program of the National Oceanic and Atmospheric Administration’s National Marine Fisheries Service (Silver Spring, MD). Standard reference material (SRM 1945) was analyzed for selected PCB congeners and persistent organochlorines. Data from our laboratory were in good agreement with those for reference materials. The average of percentage deviation from the certified values was 13% (range: 0.5-20%) for organochlorine pesticides and 28% (range: 1.3-57%) for PCB congeners.

Statistical Analyses
Analyses of were performed using EXCEL 2000, R (S7), and http://www.quantitativeskills.com/sisa/. The criterion for significance set at p=0.05.

For the model, we assumed that in this cohort of sexually immature seals measuring <1.05 m
neither sex nor age affected the log$_{10}$ contaminant values, but that these would be negatively affected by blubber thickness. Therefore, to examine the effect of time (stranding year) on contaminant levels we first log$_{10}$ transformed these. We fitted a general linear model with the log$_{10}$-transformed values as the response value, stranding year as the predictor, and blubber thickness (mm) as confounder. Terms were progressively removed from the full model to achieve the minimum adequate model (MAM), as judged by the Akaike Information Content (AIC) value, using the function STEP.

For comparison with levels found other studies since the 1970s, log$_{10}$ transformed mean values of three organochlorine compounds in blubber of juvenile harbour seals were used. Minimum sample size was set at 3. Two criteria were used to select juvenile seals only, namely a maximum seal age of 2 years and a maximum seal length of 105 cm. Two arbitrary conversion factors were used prior to log-transformation: multiplication by 0.85 for conversion from lipid weight to wet weight values, and multiplication by 0.65 for conversion from total PCB to 7 ICES PCB.

For comparison of the PCBs, DDTs and HCB values of the seals stranded in 2002 and those of the feeding study we calculated p-values using means, standard deviations and the number of seals in each group

Supporting references

S7 R Development Core Team “R : A language and environment for statistical computing” (R Foundation for Statistical Computing, Vienna, Austria, 2006).
Baseline information for polybrominated diphenyl ethers (PBDEs) in harbour seals (*Phoca vitulina*) from the Dutch Wadden Sea, 1999 and 2002.

Submitted

Jolianne M. Rijks • Thijs Kuiken • Albert D.M.E. Osterhaus • Pim E. G. Leonards

Department of Virology, Erasmus MC, Erasmus University, Rotterdam, The Netherlands; Dutch Wildlife Health Center, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; Seal Rehabilitation and Research Center Lenie 't Hart, Pieterburen, the Netherlands; Institute for Marine Resources and Ecosystem Studies, Ijmuiden, The Netherlands.
Polybrominated diphenyl ethers (PBDEs) are flame retardants, produced commercially as three main products, pentaBDE, octaBDE and decaBDE. PBDEs have been shown to accumulate in the environment since the start of their use, and adverse health effects are found at lower levels for pentaBDE and octaBDE than for decaBDE. To provide baseline information on PBDEs, 17 congeners were determined in 25 sexually immature harbour seals (*Phoca vitulina*) that stranded in the Dutch Wadden Sea between 1999 and 2002, seals—like humans—belonging to the higher trophic levels of the food chain. Host-related features of potential influence on PBDE levels, i.e., sex, morphometric data, stranding date and location were included in the evaluation. BDE-47 was the congener present in the highest levels (mean/median/min/max: 122/83/17/500 ng per g wet weight of blubber). The levels of the other consistently detectable congeners were one (BDE-99, BDE-100, BDE-153, BDE-154+BB-153) or two (BDE-28, BDE-49) orders of magnitude lower than BDE-47 levels, and overall values of these congeners correlated positively with BDE-47 values. The maximum level of BDE-183, the main component of octaBDE, was 1.1 ng per g wet weight of blubber, and BDE-209 was below detection limits in all 25 seals. A significant inverse relation between BDE-47 levels and blubber thickness was found. Seals stranded in the Western Wadden Sea had significantly higher levels of BDE-47 than those stranded in the Eastern Wadden Sea, which seems to be correlated with the levels in the food. This study on PBDE levels in harbour seals from the Dutch Wadden Sea provides baseline values against which future trends can be assessed.

**Introduction**

Polybrominated biphenyl ethers (PBDEs) reduce the propagation of flames\(^1\). The industrial production of PBDEs and their use in products began in the early 1970s\(^\text{28}\), when manufacturers started to add PBDEs as flame retardants to polymers in construction material, means of transport, electric and electronic equipment, furniture and textiles\(^1\). There are three commercial PBDE products. PentaBDE is a mixture of tetra- to hexa-BDE congeners, mainly BDE-99 and BDE-47. OctaBDE is a mixture of hexa- to nona-BDE congeners, predominantly BDE-183. Finally, decaBDE consists of BDE-209 and small amounts of nona-BDE congeners\(^1,8,12\). The global annual demand in 2001 was estimated to be 7 500 metric tons for pentaBDE (150 for Europe), 3 790 metric tons for octaBDE (610 for Europe), and 56 100 metric tons for decaBDE (7600 for Europe)\(^16\).

PBDEs enter the environment during the production process, and during the use and subsequent disposal of the products containing them\(^25\). In the decades following the start of their industrial production, PBDE levels increased in the environment\(^8\). Toxicology studies indicated that commercial pentaBDE and octaBDE caused adverse effects at lower doses than decaBDE\(^5,12\). Because of these monitoring and toxicological findings, the use of commercial penta- and octaBDE is now limited or prohibited in some geographical areas, e.g., since August 2004 the use of commercial pentaBDE and octaBDE is prohibited in all applications for the EU market.
Continued monitoring of PBDE levels in biota therefore remains necessary. The harbour seal (*Phoca vitulina*) is an indicator species for levels of organohalogens in the marine environment in Europe because, like humans, it is a top predator in the food web and a main predator in the Wadden Sea ecosystem. In the Dutch marine environment, levels of PBDEs have been measured in sediment, invertebrates, fish and birds\(^\text{3,7,14,27}\), but to our knowledge only in three harbour seals\(^6\). In this study, we determined the levels of 17 BDE-congeners in the blubber of 25 harbour seals found in the Dutch Wadden Sea between 1999 and 2002. These data should form a suitable set of baseline values against which future trends in PBDE levels in seal tissues from this region may be compared. To enable such comparisons, we have included host-related features of potential influence on PBDE levels, i.e., sex, morphometric data, stranding date and location.

**Materials and methods**

**Sample collection**

Blubber samples were taken during necropsy from carcasses of harbour seals found in the Dutch Wadden Sea from 1999 to 2002. During necropsy, the seal species and sex were determined. The carcass was then weighed to 0.1 kg. Subsequently, the carcass was placed into dorsal recumbency. Body length measured as the straight distance from the tip of the nose to tip of tail measured alongside the carcass. A ventral incision was made from mandible to anus, through the skin and the entire blubber layer. Blubber thickness was measured using a ruler graded in mm. A second incision was made laterally from sternum tip along the posterior end of the rib case and the skin with blubber was prepared away from the underlying muscle layers, taking care not to leave any blubber behind. Lateral (and left) to the sternum, a piece of skin and blubber of approximately 10 cm long and 5 cm wide was then removed, wrapped into aluminium foil and stored at -20°C. Further details on these and other necropsy procedures to determine of the cause of death of harbour seals have been recorded elsewhere\(^15\).

**The sample set**

The blubber samples came from 25 sexually immature harbour seals with an average body length of 95 cm (males: range 72 cm to 114 cm; females: range 76 cm to 111 cm). Sexually immature seals were selected because such a sample can be readily obtained in subsequent years for monitoring purposes, it reduces the effect of duration of environmental exposure to PBDEs (age) and avoids the effect of PBDE elimination through lactation (sex) on the PBDE levels measured. Samples were further selected to include both sexes (12 females and 13 males).
Baseline information for PBDEs in harbour seals from the Dutch wadden sea, 1999 and 2002.

and the different years of stranding (2 in 1999; 5 in 2000; 5 in 2001; 13 in 2002, including 8 animals that died during an outbreak of phocine distemper) (Table 1). The stranding locations are represented in Figure 1.

Figure 1: Map of the Netherlands indicating the islands or the provinces in which the 25 seals stranded.
Baseline information for PBDEs in harbour seals from the Dutch wadden sea, 1999 and 2002.

Table 1: Stranding data and characteristics of the seals in the sample and the levels of BDE-congeners measured in their blubber. Seals n° 13 to 20 were infected with phocine distemper virus.

<table>
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<th>Seal N°</th>
<th>Date found</th>
<th>Locality found</th>
<th>Sex</th>
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<th>BW (kg)</th>
<th>BT (mm)</th>
<th>BDE-congener (ng/g wet weight)</th>
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<td>F</td>
<td>0.87</td>
<td>17</td>
<td>0.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>22</td>
<td>13/08/2000</td>
<td>Terschelling</td>
<td>M</td>
<td>0.91</td>
<td>32</td>
<td>0.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>23</td>
<td>31/01/2001</td>
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<tr>
<td>24</td>
<td>11/12/1999</td>
<td>Terschelling</td>
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<tr>
<td>25</td>
<td>25/10/2000</td>
<td>Friesland (East)</td>
<td>F</td>
<td>1.11</td>
<td>33</td>
<td>0.5</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

nt: Not tested  
BL: Body length  
BW: Body weight  
BT: Blubber thickness
Baseline information for PBDEs in harbour seals from the Dutch wadden sea, 1999 and 2002.

Measurement of PBDE levels
To determine levels of PBDEs in seal blubber, about 1 g of this blubber was weighed, dried with sodium sulphate (Merck, Darmstadt, Germany) and stored for two hours. Pressurized liquid extraction was performed using an ASE300 equipment (Dionex, Sunnyvale, CA, USA) with 34 ml cells. The cells were filled from bottom to top with 33% H$_2$SO$_4$ deactivated silica gel (10 g), the dried sample, and sodium sulphate. One ml of a mixture of internal standards (BDE-58, BDE-116, and 13C-labeled deca-BDE) was added to the cells. After addition of 1 ml of toluene, and evaporation with nitrogen to 1 ml, the extract was analysed with gas chromatography (GC) coupled to a mass spectrometer (MS). The instrument was run in ECNI mode using methane. A DB-5 GC column 15 m, 0.25-mm i.d., 0.1-µm film thickness (J&W Scientific Inc., Folsom, CA, USA) was used. Helium was used as carrier gas. Peak identification was based on the retention times and the bromine clusters of m/z 79, 81 and 486.7, 488.7. For more details on the GC-MS instrumentation see de Boer et al (2003). For each congener, the detection limit was defined as three times the noise level.

Statistical analysis
To examine the relation between BDE-congener levels in individuals, levels of the BDE-congener with the highest average level (i.e., the dominant congener) were log$_{10}$-transformed. The values obtained for each individual were then plotted against the values of the log$_{10}$-transformed levels of the remaining BDE-congeners. BDE-congeners that could not be determined with precision for most of the set sample because their levels were frequently below the detection limit were excluded from this analysis.

Blubber thickness has been shown to affect organohalogen levels. Therefore we regressed the log$_{10}$-transformed dominant BDE-congener values over blubber thickness. Stranding location has also been shown to be relevant for PBDE levels in biota. Therefore we regrouped the seals that stranded in the Wadden Sea into two categories (1 = the Western Wadden Sea, including Terschelling, Vlieland, and the west of mainland Friesland; 2 = the Eastern Wadden Sea, including Ameland, Schiermonnikoog, mainland Groningen, and the east of mainland Friesland) and performed a t-test over the log$_{10}$-transformed dominant BDE-congener values. Subsequently, to determine whether there was an interaction between the effects of blubber thickness and stranding location, a general linear model was fitted in R with the log$_{10}$-transformed values of the BDE-congener as the response, and blubber thickness, location of stranding (2 levels, 1 = Western Wadden Sea, 2 = Eastern Wadden Sea), and their interaction as the predictors. Terms were progressively removed from the full model to achieve the minimum adequate model (MAM), as judged by the Akaike Information Content (AIC) value, using the function STEP. Sex and of age were not included as parameters in the model because we assumed no effect of these parameters due to the sample selection procedure. Time was not included in the model because of the short time period examined. All analyses were performed using EXCEL 2000 and R, and the criterion for significance set at $P \leq 0.05$. 

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Baseline information for PBDEs in harbour seals from the Dutch wadden sea, 1999 and 2002.

Results

PBDE values

BDE-47 had by far the highest levels, on average 122 ng/g wet weight of blubber. This was followed by BDE-99 and BDE-100, and BDE-153 and BDE-154 + BB-153, with averages between 11 and 14 ng/g wet weight of blubber. BDE-28 and BDE-49 were present in low levels, less than 3 ng/g wet weight of blubber (Figure 2, Table 1). BDE-47 represented 42% to 75% of the sum of the congeners BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154 + BB-153, when these were all determined (n=18). The remaining 10 BDE-congeners were below their detection limit, either often (BDE-66, BDE-75, BDE-77, BDE-119, BDE-138, BDE-183) or always (BDE-71, BDE-85, BDE-190, BDE-209). The maximum value for BDE-183, the main component of commercial octaBDE, was 1.1 ng/g wet weight of blubber, and BDE-209, the main component of commercial decaBDE, was not found in harbour seal blubber (Figure 2, Table 1).

Figure 2: Mean ± s.e. levels of 17 BDE-congeners in harbour seals that stranded along the Dutch coast between 1999 and 2002 (n=25, except for BDE-153 where n=19). For the 10 congeners with levels often or always below the detection limit, marked with “*”, calculations were performed using detection limit as value.

The median values of the seven BDE congeners with the highest levels were 13% to 43% below the average values, indicating that the majority of the seals had levels below average. Log₁₀ BDE-47 levels correlated significantly and positively with log₁₀ levels for each of the other six BDE-congeners (n=25; log₁₀ BDE-28, P = 2x10⁻⁵; log₁₀ BDE-49, P = 9x10⁻³; log₁₀ BDE-99, P = 4x10⁻⁵; log₁₀ BDE-100, P = 1x10⁻⁶; log₁₀ BDE-153, P = 6x10⁻³; log₁₀ BDE-154 + BB-153, P = 5x10⁻⁴; Figure 3).

Host-related factors and PBDE values

Blubber thickness in the sample was on average 17.2 mm, ranging from 6 to 30 mm. There was a significant inverse relation with the log₁₀ –transformed values of the dominant congener and blubber thickness: as blubber thickness decreased, log₁₀ BDE-47 values increased (n = 25, P = 2x10⁻⁵; Figure 4).
Baseline information for PBDEs in harbour seals from the Dutch wadden sea, 1999 and 2002.

Figure 3: The level of $\log_{10}$BDE-47 in harbour seal blubber was a good indicator for BDE-congener levels in general: $\log_{10}$BDE-47 levels correlated significantly and positively with $\log_{10}$ levels for each of the other six consistently detectable BDE-congeners.

Figure 4: The level of $\log_{10}$BDE-47 in harbour seals decreased significantly as blubber thickness increased.

Eighteen seals stranded in the Western Wadden Sea and seven in the Eastern Wadden Sea. The $\log_{10}$BDE-47 levels in seals from the Western Wadden Sea (mean ± s.d.: 2.1 ± 0.3) were significantly higher than those from the Eastern Wadden Sea (mean ± s.d.: 1.6 ± 0.3; $t$-test assuming unequal variances, $P = 0.004$).

The model including both blubber thickness and stranding location as explanatory variables for the observed $\log_{10}$BDE-47 levels found that both blubber thickness and stranding location were significant terms, both going in the directions expected from the regression and the $t$-test (blubber thickness, $b = -0.036$, $P = 9 \times 10^{-5}$; stranding location east compared to west, $b = -0.266$, $P = 0.017$). It explained 66% of the variation in $\log_{10}$BDE-47 levels, and was a better predictive model than the one which included the interaction term. This shows that the observed effects of blubber thickness and stranding location are not due to a relation between these two variables, e.g., the higher levels observed in the seals found in the Western Wadden Sea were not due to these seals having thinner blubber layers.
Discussion

Our analysis of 17 BDE-congeners in the blubber of 25 young harbour seals that stranded along the Dutch coast between December 1999 and July 2002 showed that BDE-47 was the dominant congener, representing up to 75% of the BDE-congeners, whereas BDE-209 levels were below the limit of detection. The congener pattern is in line with results for fish and marine mammals in aquatic environments in other studies but not with results for sediment samples. In sediment samples from Wadden Sea and North Sea, BDE-209 is often the dominant congener, consistent with the commercial PBDE product demand in 1999 which was far greater for decaBDE than for pentaBDE. A number of processes may contribute to explaining the discrepancy between sediment and harbour seal blubber profiles. First, there are differences in absorption and elimination of BDE-congeners in biota, with BDE-47 tending to be absorbed better and eliminated more slowly than BDE-209. Second, there is the effect of debromination. This can take place in the host species, e.g., there is evidence for debromination of BDE-99 into BDE-47 in common carp (Cyprinus carpio) and of BDE-209 into hexa- to nona-BDE-congeners in common carp and rainbow trout (Oncorhynchus mykiss). Debromination can also take place in the environment, e.g., debromination of BDE-209 in sunlight. However, the accumulation of congeners has also been shown to differ between host species and between the marine and the terrestrial environment, and is not yet fully understood.

The average level of BDE-47 in the seals was 122 ng/g wet weight of blubber (median 84) and of the BDE-99 congener 14 ng per g wet weight of blubber (median 8). In a study published in 1998, three juvenile harbour seals from The Netherlands had average BDE-47 levels of 893 ng per g wet weight of blubber (median 1 200), and average BDE-99 levels of 113 ng per g wet weight of blubber (median 140). These averages are higher than in our study. This may have been due to the small number of seals tested. Alternatively, it could indicate the start of general downward trend of these congeners in the food chain. Penta-BDE-derived congeners were levelling off between 1995 and 1997 in sediment core of 1997 from the Dutch Wadden Sea. This observation could possibly be explained by a voluntary reduction or phase-out of pentaBDE in Europe preceding the present ban. For example, industrial users in Germany agreed to phase out pentaBDE starting in 1986, and pentaBDE in Sweden was phased out in production and use by 1999.

Log_{10}-transformed BDE-47 levels correlated negatively with blubber thickness and appeared also to be affected by stranding location. The negative correlation between blubber thickness (or body condition) and organohalogen levels has been documented, and is explained by the more rapid mobilisation of fat than of pollutants in fasting harbour seals. Geographical differences in PBDE levels are well-documented. Our small sample and the possibility that seals move around in the Wadden Sea does not allow us to make a firm statement. However, given that seals are presumed to acquire PBDEs mostly through their diet, it is worth noting that...
in fish a trend for decrease in PBDE values from West to East Wadden Sea has been observed (Pim Leonards, pers. comm.).

These host-related factors (blubber thickness and geographical variation in PBDE exposure) are likely to affect the values of the other lipophilic congeners in a similar manner, as exemplified by the positive correlation between individual log_{10}-transformed values of BDE-47 and those of the other detectable BDE-congeners (BDE-28, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154 & BB-153) in individuals. Boon (2002) had previously shown that different congeners covary in the environment. The correlation was most significant between BDE-47 and the BDE-99 levels, possibly because these are the principal congeners of the commercial pentaBDE additive, and possibly also because BDE-99 may be debrominated into BDE-47.

The question remains whether the observed levels measured in blubber affect harbour seal health. An indication for a possible thyroid hormone disrupting effect was found in a study in harbour seals from around the U.K., in which comparable tissue levels of PBDEs were measured (but also other pollutants). Other toxicology studies have shown that PBDEs can affect, amongst others, behavioural and embryonic development, liver and immune functions and thyroid hormone levels. Summarizing these studies, Darnerud (2003) found a higher Lowest-observed-adverse-effect-levels (LOAEL) for commercial decaBDE (around 80 mg/kg body weight) than for commercial pentaBDE (0.6 – 0.8 mg/kg body weight) or commercial octaBDE (2 mg/kg body weight). This is reflected in the current regulations, banning the use of the latter two products. However, these toxicology studies measured the level of PBDEs ingested and are, therefore, difficult to relate to tissue levels. Also, host species vary in their ability to absorb, metabolize and excrete BDE-congeners and their sensitivity to PBDE intoxication. Understanding whether the observed PBDE tissue levels have an effect on harbour seal health is further complicated by the possible interactions between PBDEs and other pollutants, such as polychlorinated biphenyls, even though the distribution of PCBs and PBDEs in the environment do not appear to fully coincide. Given the potential negative effect of PBDEs on harbour seal health, and their position at the top of the food chain, continued measures to decrease environmental PBDE levels are warranted, as well as continued monitoring in harbour seals to assess the effects of these measures.

**Acknowledgements**

We are grateful to the people who helped collect and necropsy of the seal carcasses stranding along the Dutch coast, and the personnel of the Seal Rehabilitation and Research Center Lenie’t Hart. We thank Sicco Brandsma (IMARES) for the PBDE analysis.
Reference List


Host genetics
Heterozygosity and lungworm burden in harbour seals 
(*Phoca vitulina*)

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Jolianne M. Rijks • Joseph I. Hoffman • Thijs Kuiken • Albert D.M.E. Osterhaus • William Amos

Department of Virology, Erasmus MC, Rotterdam, The Netherlands; Dutch Wildlife Health Centre, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; Laboratory of Virology and Immunology, Department of Zoology, University of Cambridge, Cambridge, United Kingdom.
In several studies, heterozygosity measured at around 10 microsatellite markers correlates with parasite load. Usually the effect size is small, but while this may reflect reality, it may also be that too few markers are used, or the measure of fitness contains too much error to reveal what is actually a much stronger underlying effect. Here, we analysed over 200 stranded harbour seals (Phoca vitulina) for an association between lungworm burden and heterozygosity, conducting thorough necropsies on the seals and genotyping the samples obtained for 27 microsatellites. We found that homozygosity predicts higher worm burdens, but only in young animals, where the worms have the greatest impact on fitness. Testing each locus separately, we found that a significant majority reveals a weak but similar trend for heterozygosity to be protective against high lungworm burden, suggesting a genome-wide effect, that is, inbreeding. This conclusion is supported by the fact that heterozygosity is correlated among markers in young animals but not in otherwise equivalent older ones. Taken as a whole, our results support the notion that homozygosity increases susceptibility to parasitic infection and suggest that parasites can be effective in removing inbred individuals from the population.

Introduction

The publication of the measure mean d-squared, an estimator of microsatellite allele similarity, and the demonstration that it predicts aspects of fitness in deer and seals\(^9,11\) stimulated renewed interest in the link between heterozygosity and fitness\(^12\). Subsequent studies have developed both new and more effective measures for estimating heterozygosity\(^4,5,10\), and confirmed that panels of as few as 10 presumed neutral microsatellite markers often reveal statistically significant correlations with fitness. Over the last decade, the list of fitness traits found to be associated with heterozygosity has expanded greatly, from the initial analyses based on juvenile survival\(^9\), now to cover parasite susceptibility\(^10\), reproductive success\(^26,40\) and even behavioural traits, such as territory size\(^38\). Such apparent ubiquity makes these heterozygosity-fitness correlations (HFCs) a potentially important component of natural selection and suggests that understanding their prevalence and basis could help elucidate a number of evolutionary processes.

Some of the strongest HFCs have been recorded in studies of parasite load\(^10\) and infectious disease. In rehabilitating sea lions, all classes of sick animals revealed elevated homozygosity\(^1\), whereas in studies of infectious disease, heterozygosity has been implicated separately in both viral\(^42\) and bacterial\(^1\) diseases, as well as influencing the strength of the innate immune response\(^22\). However, the exact mechanism underlying these correlations remains obscure\(^21,35\). On the one hand, many authors have invoked inbreeding depression, arguing that heterozygosity at neutral markers reflects genome-wide heterozygosity, which in turn varies with inbreeding coefficient. On the other hand, both theory and simulations suggest that individuals with detectably non-zero inbreeding coefficients are usually too rare in nature to create HFCs, occurring only in small, isolated populations or species with strong polygyny\(^6,39\).
Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*).

Here, we examine the relationship between heterozygosity and the macroscopic presence of lungworm infection in harbour seals (*Phoca vitulina*). Lungworm infection is an important cause of morbidity and mortality in harbour seals, in particular in young seals. Lungworm infection is caused by two macro-parasites, often found concurrently: *Otostrongylus circumlitus*, a large species whose adults are easily visible in the principal airways and, *Parafilaroides gymnurus*, a smaller species found in the alveoli and smaller bronchioles. For the analysis, we used 27 polymorphic loci genotyped in a sample of 204 harbour seals that stranded along the Dutch coast, taking into account the relationship between lungworm burden and age. In addition, to test whether the relationship between heterozygosity and lungworm burden is dominated by a genome-wide rather than a single locus effect, we first tested for a correlation in heterozygosity across loci, and then compared the number of homozygotes and heterozygotes at every locus among seals in which worms were observed and those in which they were not seen.

**Materials and methods**

**The sample set**

The sample set was an exhaustive selection of tissue samples obtained from seals that stranded and died in the Dutch Wadden Sea in the period 1997-2003, whose death was associated either with lungworm infection (n = 26), phocine distemper virus infection (PDV; n = 169) or trauma or drowning in fishing nets (n = 9), and for which relevant data were available (Figure 1). The necropsies were performed following the procedure detailed in Kuiken and Baker (1991). This included the registration of available stranding data and clinical information, specifically: determination of species and sex, standard measurements including nose-to-tail body length, and description of gross lesions. During gross necropsy, the bronchi and lung tissue were incised and examined visually for the presence of lungworms and associated lesions. Where necessary, samples for histology, virology, bacteriology, parasitology and toxicology were collected and tested to determine the probable cause of death. Death from lungworm infection or noninfectious causes of death was established based on gross lesions, presence of and numbers of parasites, nutritional state and stomach contents. Death from PDV infection was established by gross lesions and confirmed by either reverse-transcriptase polymerase chain reaction or IgM serology.

The samples used for DNA extraction were mostly of kidney tissue, initially stored frozen at –20°C and –70°C, later transferred to 96% ethanol and stored at room temperature. In the absence of a kidney sample, lung, spleen or blood was used. A small number of kidney samples (n=13) were stored in lysis buffer (6 M Guanidine-HCL, 10 mM Urea, 10 mM Tris-HCl, 20 % Triton-X100 (v/v), pH 4.4) at –20°C and at room temperature. DNA was extracted using an adapted Chelex protocol.
Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*)

**Figure 1**: Spatial distribution and probable cause of death of the seals in the sample set. The diameter of the pie chart corresponds to the number of seals stranded at a particular location and the colours in the pie chart indicate the probable cause of death: death associated with lungworm infection (black), phocine distemper infection (grey), or trauma or by-catch (white).

**Genotyping**

Microsatellite genotyping, scoring and data entry were conducted as described previously\(^{25}\). Briefly, PCR reactions were carried out in 10 µl reaction volumes containing 1 µl template DNA, 1x Thermalase buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.1% Tween 20, 0.1% gelatine, 0.1% IGEPAL), 60 mM tetramethylammonium chloride (TMAC), 2.5% formamide, 0.1 mM dGTP, 0.1 mM dATP, 0.1 mM dTTP, 0.02 mM dCTP, 4 pmol of each primer, 0.25 units of Taq polymerase and 0.01 µCi [α\(^{32}\)P]-dCTP. Loci were amplified using the following PCR profile: one cycle of 120 s at 94°C, 45 s at T1, 50 s at 72°C; 10 cycles of 30 s at 94°C, 45 s at T1, 50 s at 72°C; 25 cycles of 30 s at 89°C, 45 s at T2, 50 s at 72°C; and one final cycle of five min at 72°C. For the majority of loci, T1 was 46°C and T2 was 48°C. However, for Pv3, Hg6.1, OrrFCB7 and OrrFCB8, T1 and T2 were 50°C and 55°C respectively. PCR products were resolved by electrophoresis on standard 6% polyacrylamide sequencing gels and detected using a phoshorimager (Fujifilm BAS-2500). Genotypes were scored by two independent observers (JMR and JIH), and entered manually into a Microsoft Excel spreadsheet. Genotypes were tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP (http://genepop.curtin.edu.au/)\(^{36}\). For each test, we set the dememorization number to 10 000, the number of batches to 1000 and the number of iterations per batch to 10 000.

**Calculation of heterozygosity**

Several alternative versions of heterozygosity have recently been proposed, all of which aim to produce a measure that correlates most strongly with \(F\), the inbreeding coefficient. These...
measures include methods to compensate for missing genotypes [standardised heterozygosity, SH\(^{10}\)], to exploit the evolutionary similarity of different alleles [mean d-squared\(^{11}\)] and to use allele frequencies to weight scores according to the alleles in the genotype [internal relatedness, IR\(^{4}\)] or the diversity of the loci being scored [heterozygosity weighted by locus diversity, HL\(^{5}\)]. Which of these performs best depends on several factors, including the completeness of genotyping, the diversity of the loci being scored and the range of inbreeding coefficients, F, represented in the sample. However, mean d-squared is now seen as performing relatively poorly unless two genetically distinct populations have recently mixed\(^6\) whereas SH tends to perform marginally less well than IR across a range of scenarios\(^4,6\). Heterozygosity weighted by locus HL is a recently proposed method that has yet to be used widely, but simulations suggest that it can outperform IR, particularly at loci with higher allelic diversity\(^5\). In view of the above, we chose to use both IR and HL to conduct a comparison.

**Data analyses**

Seals were categorized as ‘infected’ or ‘uninfected’ depending on the presence or absence of lungworms in the respiratory tract at gross necropsy. Using sex and body length to estimate age\(^{33}\), males up to 95 cm and females up to 90 cm were considered to be up to 1-year-old, and categorized as ‘young’ in this paper; the remaining seals referred to as ‘older’.

The relationship between heterozygosity and lungworm burden was first analysed by comparing the mean of the measure of heterozygosity (HL, IR) of all infected seals (n = 54) to that of all uninfected seals (n = 150; t-test). Then, as infection and mortality due to high worm burden is most likely to occur in the first year of life, we performed the same analysis for young seals (total n = 43; 29 infected, 14 uninfected; t-test). To verify that the reduction to this age category was justified, we checked the significance of the interaction between age category (young, older) and lungworm burden (uninfected, infected) by performing a univariate analysis of variance on a model with the measure of heterozygosity (HL, IR) as dependent variable and age category (young, older) and lungworm status (uninfected, infected) as explanatory variables. These analyses were performed in SPSS.

Finally, any relationship between heterozygosity and lungworm burden could be due either to a genome-wide (inbreeding) effect or to a single locus effect caused by chance linkage between one or more of our markers and a gene experiencing balancing selection. To test which is the more likely mechanism in the current dataset, we tested whether heterozygosity was correlated across loci using the method of Balloux et al. (2004)\(^6\). Here, the loci are divided randomly into two equal groups and then a correlation coefficient calculated across individuals between the paired heterozygosity estimates, one from each group of markers. By repeating this process 100 times, each time dividing the markers into different groupings, one can assess the robustness of any correlation present. A robust positive correlation suggests that one or more inbred individuals are present in the group, with stronger correlations suggesting greater numbers of
Heterozygosity and lungworm burden in harbour seals (Phoca vitulina)

inbred individuals and higher F-values. We applied this test to young animals (n = 43) and older animals (n = 161) separately. In addition, we used a Fisher’s exact test to ask whether, at each locus in turn, there was a difference in heterozygosity between infected and uninfected young individuals. If one or a small number of loci contribute to any effect found, these should yield an equivalent number of significant tests. However, under a genome-wide effect we expect to find that most loci reveal similar weak trends for heterozygotes to be uninfected.

Results

Microsatellite locus selection
We considered 94 published pinniped microsatellite primers. Of these, 38 had previously been tested on four harbour seals from Scotland and were dismissed because they were either monomorphic or failed to amplify. The remaining 56 loci were tested on a panel of 43 Dutch harbour seals, revealing 30 that were polymorphic (Table 1), 19 that were monomorphic and seven that either gave unscorable results or failed to amplify. Three of the 30 polymorphic loci (Lw18, Pvc74 and ZwcF09) were subsequently excluded because they were not in Hardy-Weinberg equilibrium. As previously reported, Lw18 is likely to be X chromosome-linked because Hardy-Weinberg equilibrium is observed in females (females, test for HWE, $P = 0.5444$) but not in males (males, test for HWE, $P = 0.0000$). This left 27 loci, all but one of which carried six or fewer alleles in the Dutch population, the last locus (Pv3) carrying 23 alleles (four alleles on average). Following sequential Bonferroni correction to compensate for multiple statistical tests, we found no evidence that any locus exhibited significant linkage disequilibrium with any other. Observed heterozygosity varied between 0.023 (M11a) and 0.905 (Pv3), (on average 0.336), see Table 1.

Heterozygosity and lungworm burden
In the sample as a whole (n = 204 individuals), mean measures of heterozygosity did not vary significantly between the uninfected and infected seals, though the difference in means did go in the direction expected if heterozygosity increases resistance to worm infection (mean HL$_{150}$ uninfected seals = 0.512; mean HL$_{54}$ infected seals = 0.531; t-test: $P = 0.314$; mean IR$_{150}$ uninfected seals = 0.014; mean IR$_{54}$ infected seals = 0.050; t-test: $P = 0.313$). However, lungworm burden was more common in young seals (29/43 infected) than in older seals (25/161 infected), and the proportion of deaths due to lungworm was also greater in young seals (Figure 2). When only the group of young seals was considered, the average heterozygosity was significantly greater in uninfected than in infected young seals, suggesting heterozygosity does increase resistance to lungworm infection (mean HL$_{14}$ uninfected seals = 0.431; mean HL$_{29}$ infected seals = 0.543; t-test: $P = 0.006$; mean IR$_{14}$ uninfected seals = 0.114; mean IR$_{29}$ infected seals = 0.061; t-test: $P = 0.027$). The interaction between age category and lungworm burden was significant for HL but not for IR (HL univariate analysis of variance, $P = 0.012$; IR univariate analysis of variance, $P = 0.053$), validating the cut-off between age categories for HL but not quite for IR.
### Table 1: Number of alleles, observed heterozygosity (HO), expected heterozygosity (HE) and probability values for derivation from Hardy-Weinberg equilibrium (HWE P-value) at 56 microsatellite loci for harbour seals that stranded along the Dutch coast.

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<th>N° of alleles (reference in superscript)</th>
<th>N° of alleles in harbour seals</th>
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- : not tested

The allele frequencies are compared to allele frequencies found in other studies or populations.

a: Hoffman et al., 2006 & Hoffman unpublished data
b: in harbour seals of the North Sea 20
c: in harbour seals of the Western Wadden Sea 20
Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*)

Figure 2: Relative frequency of lungworm infection in the two age groups (total n = 204). Seals in which lungworms were observed macroscopically (infected) either died of lungworm infection (black areas) or phocine distemper virus infection (slanted hatched areas); seals in which no lungworms were observed macroscopically (uninfected) died of phocine distemper virus infection (white areas) or by-catch or trauma (dotted area). Seals were classified into age groups based on sex and length. Males up to 95 cm and females up to 90 cm were classified as ‘young’, all larger seals as ‘older’.

Genome-wide or single locus effects
When all adults were used to test for a correlation in heterozygosity among loci, the mean correlation coefficient was 0.018 (± 0.06 s.d.) and did not differ significantly from zero, implying that this group contains few or no appreciably inbred individuals (Figure 3). In contrast, among young animals the mean correlation coefficient was 0.213 (± 0.087 s.d.), much larger and significantly greater than zero, implying the presence of inbred individuals. In addition, when each locus in turn was tested for a difference in heterozygosity between infected and uninfected young seals, none of the P-values were significant (Fisher’s exact test at each locus). However, 21 of the 27 loci did reveal a tendency for greater homozygosity in the infected, a significant imbalance (Sign test, \( P = 0.019 \)). Such a pattern is most likely to result from a slight tendency towards heterozygote advantage affecting every locus, that is, a genome-wide or inbreeding effect, rather than one or a few loci showing a big effect.

Discussion
In this paper, we examined the relationship between heterozygosity and lungworm burden in harbour seals that stranded on the Dutch coast and died. Overall, there is no relationship between worm burden and heterozygosity. However, lungworms exert their maximum impact on fitness in young animals soon after weaning. When the analysis was repeated in this light, comparing affected young animals with all others, a significant difference in heterozygosity was found. The pattern appears to be driven by inbreeding depression, with young animals showing evidence of inbreeding not found among adults and no one marker standing out as showing a dominant contribution.
Figure 3: Correlation in heterozygosity among markers in older seals (black bars, n = 161) compared with young seals (white bars, n = 43). In each set of animals, the 27 microsatellite markers, for which the animals were typed, were divided randomly into two equal groups and the correlation coefficient, R, between the estimates for each group calculated. The bar chart below presents data for 100 replicates. Values centering on zero suggest that inbred individuals are rare or absent, while increasingly positive values suggest the presence of inbred individuals (strictly, a higher variance in the underlying inbreeding coefficients of the sample).

Our finding of a link between heterozygosity and lungworm burden in young animals but not in the data set analysed as a whole appears to reflect the life history of the parasite. Lungworms appear to infect harbour seals early in life. The mode of infection is unknown, though it is likely that infective larvae develop in fish and infect seals via the oral route\(^{32,34}\). Some young seals develop higher burdens than others and, among these, some die as a result of their infections. Generally, in domestic animals, worm burden following primary infection depends on the exposure conditions, for example parasite intake, and on host immunity, first innate and then specific. The specific immunity that develops and helps clear the primary infection also impedes the development of worms in subsequent infections\(^{41}\), as has been shown, for example, in calves infected with lungworm (Dictyocaulus viviparous)\(^{16,37}\). The development of protective specific immunity against lungworms in harbour seals has not been proven, but seals do develop antibodies against lungworms\(^{15}\) and lungworms are uncommon in older seals. Our data reflect this in that the strongest association between genotype and worm burden occurs early in life when the impact of the worms is likely to be largest.

In revealing the lungworm HFCs, we compared two measures of heterozygosity: IR, which weights allele sharing by the frequencies of the alleles in a genotype, and HL, which weights heterozygosity by the variability of each locus at which an individual is homozygous. Our results largely support the claim made by Aparico et al. (2006)\(^\text{5}\) that HL is often a ‘better’ measure, in
the sense that in most of the significant trends we find, HL generates a lower P-value than IR. We therefore endorse the use of HL as the measure of choice for studying heterozygosity-fitness correlations in natural populations, though it is still of interest to compare the two measures, since they are expected to perform somewhat differently depending on the variability of loci being used.

There is currently considerable debate concerning whether heterozygosity-fitness correlations are due mainly to genome-wide changes in heterozygosity brought about by inbreeding, or through chance associations between markers and neighbouring genes under balancing selection. Despite our rather small sample size of affected young animals, our analysis suggests that the relationship between heterozygosity and lungworm burden is dominated by a genome-wide effect, that is, inbreeding. We provide two lines of evidence. First, heterozygosity is essentially uncorrelated across loci among adult animals, but is rather strongly correlated among young animals. Such a pattern is consistent with young animals including some individuals with F-values that are high enough to detectably impact on the probability that any given marker is heterozygous. Just how many inbred individuals we have sampled cannot be determined without deploying hundreds rather than tens of markers. The second line of evidence is that, when each locus is tested separately for a relationship between heterozygosity and worm burden, no one locus stands out, but instead a significant majority reveal a trend in the direction of homozygosity conferring susceptibility. Such a pattern is consistent with a genome-wide effect.

The presence of a detectable genome-wide effect, and in particular, the large difference between adults and juveniles, is interesting in the context of how selection acts on a population through parasites and disease. It is known that harbour seals exhibit strong population substructure, with significant genetic differences between populations that are easily within the reach of dispersing youngsters. Such structure has the potential to allow or even promote some level of inbreeding. In our sample of individuals from the Dutch population we do indeed find evidence of inbred individuals, but only among the juveniles, not the much larger sample of adults. By implication, any individuals who are born to related parents are disproportionately likely to suffer lethal worm burdens, which in turn remove them from the population. This therefore agrees with earlier studies that suggested inbred individuals carry more parasites, a greater diversity of parasites and may provide a weak point through which new pathogens can enter the population.

In conclusion, we reveal a signal in the direction of reduced heterozygosity correlating with lungworm burden in young seals. Our results emphasize the importance of accurately quantifying fitness. Worm burden alone reveals little unless considered alongside measures of age and an appreciation of the age-specific effects of lungworm infection on seal morbidity and mortality.
Acknowledgements
We thank the volunteers who cover the Dutch coast in search of stranded seals; the Seal Rehabilitation and Research Centre Lenie ’t Hart for their interest in establishing the cause of death of seals that strand; Paulien Bunskoek, Fiona Read and Marco van de Bildt for assistance during sample selection; Hazel Nichols, Bill Lee and Dan Morrish for their help during the laboratory work; Kanchon Dasmahapatra for kindly providing logistical support; René Eijkemans for assistance with statistics; and the reviewers for their valuable comments.
Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*)

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Summarizing Discussion
In 1988, a massive die-off of seals took place in European waters, caused by a previously unknown morbillivirus, phocine distemper virus (PDV)\(^{13,45,53}\). The epidemic was both impressive (more than 18 000 seal deaths\(^{17}\)) and intriguing. Why did harbour seals die in such high numbers? Where had the virus come from, why did it emerge? Would the infection disappear from the population? If so, would such epidemics reoccur? What would the long-term impact of the disease be on seal populations?

Shortly thereafter several other morbillivirus species were discovered in marine mammals. Dolphin morbillivirus (DMV) was first isolated during a mass die-off of striped dolphins (\textit{Stenella coeruleoalba}) in the Mediterranean in 1990\(^{18}\), and possibly played a role in a mass die-off of the Mediterranean monk seals (\textit{Monachus monachus}) in 1992\(^{82}\). Porpoise morbillivirus (PMV) was isolated from stranded harbour porpoises (\textit{Phocoena phocoena})\(^{47}\), and sequences of a pilot whale morbillivirus (PWMV) were identified in a stranded pilot whale (\textit{Globicephala melas})\(^{74}\). Also, canine distemper virus (CDV) emerged in marine mammals (Baikal seals \textit{Phoca sibirica} in 1987\(^{25,54,85}\); Caspian seals \textit{Phoca caspica} in 2000\(^{18}\)). The epidemics caused by these viruses raised questions similar to those raised during the 1988 PDV epidemic.

Between 1990 and 2001, no mortalities due to PDV were observed in seals in European waters and sero-surveillance showed a gradual decline in numbers of seals with antibodies to morbillivirus\(^{37,75}\). This indicated the infection was not circulating in these seal populations, and, as predicted by models\(^{15,26,72}\), harbour seal (\textit{Phoca vitulina}) populations in European waters were largely susceptible to PDV at the time PDV infection resurfaced in 2002.

The 2002 epidemic was the second documented PDV epidemic in seals in European waters. Would there be high mortality among harbour seals like in 1988, or would there be less mortality since more susceptible genotypes would have been removed from the population? Would there be less immune suppression in an apparently cleaner environment? Would the pathogenicity of the PDV strain differ of that of 1988? Would there be a role for co-infections? Would the infection cause deaths among grey seals (\textit{Halichoerus grypus}) now that these were more common in the Dutch Wadden Sea? Since a substantial part of the harbour seal population died during the PDV epidemic, this offered a unique opportunity to learn more about PDV infection and other infectious or non-infectious diseases present in the population, feeding habits and ingestion of corpora aliena, exposure to environmental contaminants, and more generally the life history of the harbour seal population.

To address the above questions, all seal strandings during the 2002 PDV epidemic in the Netherlands were recorded in a central database, live seals were collected for rehabilitation and more than half of the carcasses were systematically necropsied. This thesis presents a multidisciplinary analysis of the data obtained from the above. The first three chapters deal with the epidemiology of PDV infection: a description of the 2002 PDV epidemic (\textbf{Chapter 2.1.}), a
Summarizing discussion

review of factors affecting morbillivirus transmission (Chapter 2.2.), and a stage-structured model (Chapter 2.3.). The next chapter describes the pathology, using a quantitative approach (Chapter 3.1.). The subsequent two chapters deal with toxicological data: levels of environmental contaminants in seals during the outbreak and the preceding decade (Chapter 4.1.), as well as levels of PBDE flame retardants in seals at the turn of the century (Chapter 4.2.) were determined and evaluated. The last chapter deals with seal genetics (Chapter 5.1.). To this end, data from seals that had stranded in The Netherlands before the PDV epidemic were used in addition to data from the seals that stranded during the epidemic in Chapters 3.1. to 5.1.

In conclusion, the first four chapters focus on describing and improving our understanding of PDV infection, while the later chapters have a broader scope, dealing with seal and ecosystem health in more general terms. Overall, we have evaluated the results obtained in the thesis with special emphasis on comparing the PDV epidemics of 1988 and 2002.

Epidemiology

As in the 1988 epidemic\(^{34}\), harbour seals were much more likely to die from PDV infection than grey seals\(^{30}\). The seals found stranded in the Netherlands during the 2002 PDV epidemic were virtually all harbour seals (2279 of the 2284; Chapter 2.1.), even though 500 or more grey seals were living along the Dutch coast in 2002.

As in 1988, there were striking temporal differences among stranding locations, the 2002 PDV epidemic starting and peaking at different times in different areas. The pattern of spread differed from 1988, but the location of the index case on 4 May 2002 was again Anholt\(^{30,61}\), about three weeks later than in 1988 (12 April 1988\(^{17}\)). After Anholt, PDV was identified in 2002 in the Kattegat and the Skagerrak in May; then Southern Norway and the Dutch Wadden Sea in June; the southern part of the German Wadden Sea in July; the Wash (U.K.), the northern part of the German Wadden Sea, the Danish Wadden Sea, the Southwestern Baltic in August; the Western Baltic, Limfjorden and the remainder of the U.K. and Ireland in September\(^{61}\). In the Netherlands, the index case was found three weeks later in 2002 than in 1988 (16 June 2002 versus 22 May 1988\(^{17}\)), but the date of the median case in 2002 (2 September) was similar to that of 1988 (4 September) and the epidemic duration was shorter than in 1988 (93 days instead of 115\(^{17}\); Chapter 2.1.). It is generally accepted that during early phases of disease invasion, chance events significantly influence the probability of the emergence of an epidemic\(^{44}\). That both epidemics reached their median case around the same time may be related to the fact that conditions optimizing transmission occurred around the same time in both years.

The observed temporal differences among locations were generally assumed to be due to differences in time of introduction of the PDV infection into local populations. It was more obvious in 2002 than in 1988 that two patterns of disease dispersal existed, one primarily to
adjacent locations, and the other to ‘far-off’ locations. After 2002, some authors suggested grey seals were vectors of infection to ‘far-off’ locations. First, because grey seals sero-converted during the PDV epidemics and were possibly infectious when infected; second, because grey seals were known to swim longer distances than harbour seals; third, because they mixed with harbour seals on Anholt beaches, and at the other ‘far-off’ locations. The location of the Dutch index case, Vlieland (Chapter 2.1.), does not contradict this idea, the case being found close to the tidal basin where the grey seal breeding colony is established. However, although the grey seal may be a vector, other marine or terrestrial mammals cannot be excluded at this time. For example, the grey seal hypothesis is based on normal host behaviour, but behaviour in individuals infected with morbillivirus is often altered. They may display different movement patterns than normal (Chapter 2.2.), creating the opportunity to introduce the infection into a ‘far-off’ area. Altering host behaviour to infect new susceptible populations, together with cross-species jumps and variation in virulence are important persistence strategies for viruses of the genus Morbillivirus, which otherwise tend to drastically eliminate their pool of susceptible hosts by either removal or life-long immunity. Finally, regardless of the vector, locations do not necessarily have to be infected by individuals coming from Anholt, as is often presumed. They could equally well have been infected by individuals originating from elsewhere. Possibly, isotope patterns in vibrissae of grey seals and harbour seals, including the first harbour seals that die during an epidemic, could shed some light on these issues.

In certain situations, temporal differences among locations may also be due to differences in local population sizes. We assumed that this could be the reason why the temporal differences were observed in the Netherlands between Zealand and the Dutch Wadden Sea locations. Modelling of data from 1988 had shown that the disease developed in a similar way in herds between 80 and 1500 individuals. However, the number of harbour seals in Zeeland was at the lower end of this range, with less than 100 harbour seals and only a handful of grey seals counted in the whole Delta area in the summer of 2001. We found that the median stranding date in Zealand was nearly one month later than that of locations in the Dutch Wadden Sea (Chapter 2.1.). We suggested that, because there were fewer seals in Zealand and that they were more widely dispersed than in the Wadden Sea, contact rates among them were lower (Chapter 2.1.), increasing the role of chance events in the start and dynamics of the epidemic.

During the 2002 epidemic the calculated overall mortality in harbour seals in European waters was again extremely high (47%), albeit lower than in 1988 (57%). In 2002, there were 22 500 stranded seals, corresponding to approximately 30 000 seal deaths. Between locations, the estimated cumulative mortalities in 2002 differed even more extremely than in 1988, ranging in 2002 from 1% in Scotland to 66% in the Skagerrak. We found that in the Netherlands, cumulative mortality was very similar in both epidemics (53% in 1988, and 54% in 2002), and at the high end of the range (Chapter 2.1.).
Three factors that may cause differences in cumulative mortality among locations and epidemics are differences in exposure, virus virulence, or host susceptibility to fatal disease after infection. We discuss the factors for the PDV epidemic in the Netherlands. Exposure probability and frequency varies with seal behaviour and density. Exposure probability may be less if the infection only reaches an area when breeding and moulting activities are coming to an end. In the Netherlands this was not the case, because the introduction of PDV both in 1988 and in 2002 occurred early in the season, at the start of breeding and moulting activities. Exposure frequency may have had an effect on the severity of the disease. This has been suggested for measles where secondary measles cases in a household were shown to have a higher death risk than the primary cases, although this has not yet been explored experimentally. Data on possible differences in virus virulence among locations and epidemics are largely lacking: virus gene fragments have been sequenced but no comparative experimental study to determine virulence has been carried out. Sequencing of a P-gene fragment showed 99% homology between viruses of Danish and Dutch cases in 2002 and more than 97% homology between 1988 and 2002 isolates, and sequencing of the H-gene 98-99% (with 98% amino acid identity). This shows that the 2002 and the 1988 PDV viruses were closely related, but because few changes in genes may be enough to change virus properties, no conclusions can be drawn from these data with regard to virus virulence.

Finally, levels of cumulative mortality are a function of host susceptibility. Host susceptibility to fatal disease may be influenced by certain pre-existing immune suppressive conditions and vitamin A deficiency. Environmental contaminants may affect the immune response and reduce vitamin A levels. In the Netherlands, we found that in 2002, the levels of polychlorinated biphenyls (PCBs) in harbour seal tissues were still within the range of those associated with immune suppression, like in 1988. Host susceptibility to fatal disease may also be influenced by co-infection with other pathogens. In the Netherlands, co-infections may not have varied significantly between 1988 and 2002: endoparasitic co-infections were similar, and the most common bacterial co-infection in 2002, Bordetella bronchiseptica, is considered a nasal commensal in healthy hosts, and therefore likely to have been prevalent in 1988. Further, host susceptibility is influenced by specific immunity. Seals aged 14 years or older hardly stranded in 2002 in the Netherlands, presumably because they were immune survivors of the 1988 epidemic. However this age category represents only a small fraction of the population. Host susceptibility could also have been influenced by genetic changes in the harbour seal population. No samples from 1988 were available to address this issue. However, we did examine whether seals that died at the start of the epidemic were more inbred than seals that died later on. This had been observed with DMV in striped dolphins. Our data on seal mortality were however inconclusive. Collectively these data, together with the observation that cumulative mortality in the Netherlands did not change between the two epidemics, suggested that no major genetic change in Dutch harbour seal population or PDV had occurred between the two epidemics.
In both the 1988 and the 2002 epidemics, mortality patterns proved to be sex- and age-related. Data from elsewhere in the North Sea suggested that in contrast to subadults (1- to 3-year-olds), pups and mature seals suffered higher mortality, which was also more prominent in males than females\textsuperscript{31,32}. In our description of the 2002 epidemic in the Netherlands, subadults were clearly well represented amongst the dead. However these data should be interpreted with caution as no reliable data on sex and age distribution of the population is available.

There were significant temporal differences in stranding among sex and age categories: subadults were affected earlier than juveniles and adults, and within each age category, males earlier than females (Chapter 2.1.). Most surprising was that the disease started in June, when adult females and their pups were hauled out\textsuperscript{49,64}, yet at the beginning of the epidemic these had hardly stranded. We suggested that the observed temporal differences could be explained by sex- and age-related variations in behaviour and tissue contaminant levels. Sex- and age-related variations in behaviour may have influenced exposure rates. We took for granted that transmission would be most likely if there was close contact between the infected and the susceptible hosts (Chapter 2.2.) and brought forward several behaviour patterns that could explain the observed stranding patterns: the relative isolation and reduced mobility of adult females and their pups during the first weeks after whelping (June)\textsuperscript{24,52,78}; social play and interactions among subadults (throughout summer)\textsuperscript{62,63,86}; interactions among (nearly) mature males at the beginning of the breeding season (beginning July) and between mature males and females during the breeding season (July-August)\textsuperscript{71,77}; and increased haul out during moult, moult occurring earlier in subadults than in adults\textsuperscript{76}. Sex- and age-related variations in contaminant burdens may have influenced susceptibility. Adult females and juveniles may have been less immune suppressed than adult males and subadults, because adult females tend to lose contaminants through lactation and whelping, and juveniles have had less time to accumulate contaminants\textsuperscript{1}. As a result, adult females and juveniles may have succumbed more slowly to PDV infection (Chapter 2.1.). Ultimately, behavioural explanations seem more likely. Our pathology results, for example, suggest that PDV infection is not more protracted in juvenile seals (Chapter 3.1., cf. below Pathology).

Because we found temporal differences in stranding among sex and age categories, we decided to create a model to see if stage-structured transmission is supported. We used the stranding incidence data and a Susceptible-Infected-Removed model applying a WAIFW (« Who Acquires Infection From Whom ») matrix to model three situations, from a complete lack of stage structure with homogeneous mixing to strong heterogeneous mixing. Adding stage structure provided a better description of the data. Overall, the model with strong heterogeneities was the best-fit model, suggesting that the harbour seal population in the Dutch Wadden Sea transmitted PDV in a stage-dependent manner during the 2002 epidemic. The transmission estimates generated by the model indicated that transmission between juveniles and adults had been the lowest, whereas transmission within subadults
and between adults and subadults had been the highest. Some estimates were correlated, and could therefore take on several values. The basic reproductive ratio $R_0$ was estimated to be 3.08, thereby falling within the range of other $R_0$ estimates for PDV. The WAIFW matrix does not distinguish between seal contact structure and mechanisms of epidemic spread and besides behaviour there may still be other factors affecting stranding pattern, as discussed previously.

The model made use of stranding incidence data. This was possible because around three-fourths of the seals that died stranded, and many of them could be recovered. This is a rather exceptional situation in wildlife die-offs. It is probably the result of the topographical characteristics of the Wadden Sea, Delta area, and North Sea (all fairly enclosed water bodies), seal carcass buoyancy (due to the blubber layer, emphysema, and post-mortal bloating), and absence of large scavengers.

In the Dutch Wadden Sea, we were also able to identify spatial differences in stranding among age categories, consistent with known differences in age distribution among areas. High proportions of juvenile and adult seals stranded in the province of Groningen, consistent with the presence of a core breeding area located there; high proportions of subadults stranded in the western part of the Dutch Wadden Sea, consistent with the migratory influx of young animals to this area; and the highest density of strandings occurred on Schiermonnikoog, consistent with the summer distribution of harbour seals in the Dutch Wadden Sea. The fact that these spatial differences could be explained well was encouraging, because there are of course confounding factors that affect the temporal and spatial stranding patterns observed. In particular, we showed that wind force and direction clearly affected the number of seals found stranded on a given day during the epidemic, and that the proportion of stranded carcasses that was decomposed increased with time during the epidemic.

The questions ‘Where did the infection come from?’ and ‘Where was it in between epidemics?’ remain unsolved. The original source of the epidemics is still thought to be located in the Arctic or the North-west Atlantic, because serological studies had indicated that over lengthy periods of time substantial numbers of individuals of several species of carnivores from these areas had antibodies against morbillivirus infections (probably PDV). In addition, there is evidence to suggest PDV-associated seal deaths in the western Atlantic, although no virus has been isolated from these areas.

Terrestrial virus-host pairs have shown to have one or two reservoirs, which are susceptible to both infection and disease. Therefore, grey seals cannot be considered a likely reservoir host, because they are barely susceptible to disease from PDV infection. Population threshold levels for viral persistence are often difficult to determine for wildlife. A number of factors may affect the threshold: a second reservoir host species, variation in virus virulence, individuals with
extended infectiousness, or viral persistence in the environment. Many of the characteristics of morbillivirus species virulence as described in Chapter 2.2. could be explained by the existence of quasi-species. As Vignuzzi et al. (2006) wrote, this theory states that a virus is not just a collection of diverse mutants but a group of interactive variants, which together contribute to the characteristics of the population. With regard to the role of individuals with extended infectiousness, it is unclear how infectious they may be over time. With regard to persistence in the environment, low temperatures, low relative humidity and long dark winter season as observed in the Arctic may contribute to persistence of virus infectivity in substrates and carcasses (Chapter 2.2.).

Pathology

Necropsy was performed on 1315 of the 2284 seals found stranded in the Netherlands in 2002. To examine the effects of different factors on the pathology of phocine distemper, we used a sample of 369 harbour seals, namely the cases that were not decomposed. We performed laboratory tests on these (RT-PCR, IHC, IgG serology, IgM serology, or a combination of these), and used the 232 confirmed cases for describing lesions, state of nutrition, co-infections, and for determining correlations between assay results, lesions, age and co-infections. None of the five grey seals that had stranded in the Netherlands in 2002 tested positive in laboratory tests.

During the 1988 PDV epidemic the most common lesions at gross necropsy were pneumonia and interstitial emphysema. These lesions were also the main gross lesions observed in PDV-infected harbour seals in 2002 in the Netherlands and elsewhere in 2002 (Chapter 3.1.). Pneumonia, observed in 94% of the cases, was characterised microscopically as bronchopneumonia or broncho-interstitial pneumonia. Emphysema occurred in 86% of the cases. We supposed that emphysema developed as a result of inflammation and airway obstruction, the well-developed interlobular septa of seals impeding collateral ventilation. As a new finding, we showed significant age-related differences in the extent and severity of pneumonia and emphysema: extra-thoracic emphysema was rare in <1-year-olds compared with older seals, even though severe pneumonia was more common. We found two possible explanations. The first is protracted pneumonia in older seals, allowing the development of extra-thoracic emphysema. The second is limited diving capacity in young compared to older seals, the latter more likely to perform dives that exacerbate the development of interstitial emphysema.

Another result possibly pointing towards either an age-related difference in disease length or an age-related difference in development, is the fact that seals ≥ 3-years-old were significantly more often IgG positive than younger seals. This could fit with earlier mortality among young seals or age-related compromised immune responses in young seals.

There were few seals with other significant gross lesions in the respiratory tract or thoracic cavity.
(9%), and few seals with significant gross lesions in other organ systems (7%). The last group had significantly less pneumonia and emphysema and were significantly more IgG positive than the remainder of the PDV cases, which suggested that these seals had a different (lengthier) disease course.

As mentioned previously, the occurrence of ectoparasitic, helminthic, viral and bacterial (often *Bordetella bronchiseptica*) co-infections was common both in 1988 and 2002. In 2002 in the Netherlands, it was striking that macroscopic evidence of suppurative pneumonia was rare, but it was commonly diagnosed histologically. The explanation could be that a high proportion of the bacterial infections in the lung were peracute to acute and had not led to macroscopically visible changes. In the future, it could be interesting to examine for presence of protozoa, because these may be latently present, evolve in immune suppressed seals, and be potentially zoonotic.

Several other points were raised by this study. First, seals positive by RT-PCR in the brains tended not to be positive by RT-PCR in the lungs, possibly because they were able to clear the virus from all tissues but the brain. These results indicate that both lung tissue and brain tissue need to be tested by RT-PCR when PDV is suspected. If opening the cranium to sample the brain poses logistical difficulties, an alternative is to obtain a brain sample by inserting a straw through the foramen magnum, as is done for rabies testing. Second, PDV-infected seals possibly suffer from severe dehydration. PDV-infected seals, especially larger ones, had significantly greater kidney and adrenal weights than seals that died of other causes. PDV infection is likely to cause seals to lose water and electrolytes excessively through the secreta and excreta, and the nearly consistently empty stomachs indicated PDV-infected seals were impeded in their water intake from prey. This question of dehydration could be addressed through measurement of urea nitrogen in vitreous humour fluid. Third, we had hoped to learn more about seal diet, but virtually all the stomachs were empty, except for corpora aliena. With regard to the latter, a few seals had ingested sport fishing devices resulting in severe internal damage. Sport fishers should be made aware of such findings.

**Toxicology**

We used blubber samples of 45 sexually immature harbour seals, that had stranded during the 2002 epidemic and the decade before, to evaluate trends for levels of organochlorine compounds (OCs), namely polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT), tris(4-chlorophenyl)methanol (TCPMOH), chlordane compounds (CHLs), tris(4-chlorophenyl)methane (TCPMe), heptachlor epoxide (HP-epox), hexachlorocyclohexane (HCHs) and hexachlorobenzene (HCB), as well as for the newly introduced flame retardant polybrominated diphenyl ethers (PBDEs). Another 25 sexually immature harbour seals (*Phoca vitulina*) that stranded in the Dutch Wadden Sea between 1999 and 2002 were
used to get more information on PBDE levels in harbour seals from the Dutch Wadden Sea. Harbour seals chronically exposed to environmental contaminants through their diet have vitamin A and thyroid hormone deficiency, as well as impaired immunological function. Vitamin A deficiency was recently shown to significantly affect the mortality rate not only in measles-infected humans, but also in CDV-infected ferrets (Chapter 2.2.). This suggests that vitamin A levels could be relevant to the outcome of morbillivirus infections in hosts in general; as previously mentioned, contaminant levels in combination with diet would then contribute to explaining the differences in mortality observed between locations and years.

The second point highlighted after the 1988 PDV epidemic had been that there were spatial differences in OC levels. OCs had been examined in harbour seals in multiple countries and had been at the high end of the range in the Baltic and southern Wadden Sea, and at the low end of the range in Norway and Northern Ireland. Surprisingly, to date there are few peer-reviewed articles presenting results from samples from the 2002 epidemic to determine how the levels of OCs evolved since 1988. For the Netherlands, it was hypothesized that the improvement in reproductive rate of harbour seals in the Netherlands after the 1988 epidemic was a result of selective mortality during the 1988 epidemic, which would have eliminated the adult females that had contaminant-induced sterility. It is also generally thought that harbour seals in 2002 suffered less from contaminant-induced immune suppression because of overall decreasing trends of PCBs and DDE in marine mammals in the northeastern Atlantic. In our study, after correcting for blubber thickness, we found that only HCH and HCB levels showed a significant decline in the period 1993-2002. However, all remaining OCs, including PCBs and dichlorodiphenyl-trichloroethane (DDT), did not decline significantly. PCBs remained at levels previously associated previously with immune suppression in harbour seals (Chapter 4.1.). Our results therefore indicated that, contrary to general belief and despite reduced input and declining sediment levels in the North Sea ecosystem, PCBs had stabilized in harbour seal tissues at potentially harmful levels. In other species in the higher trophic levels of the North Sea food chain, namely the gannet Morus bassanus, studies on eggs also indicated certain PCBs had remained stable or even increased. Further, there were other geographical areas were it was shown that PCB concentrations were not changing in seal tissues, as predicted by Tanabe.

A new development since 1988 had been the realisation that polybrominated diphenyl ethers (PBDEs) accumulate in the environment, and if not regulated, could lead to new environmental problems. PBDEs are flame retardants produced and used since the early 1970s. There used to be three commercial products, pentaBDE, octaBDE and decaBDE, but because adverse health effects were found at lower levels for pentaBDE and octaBDE than for decaBDE, the first two have been more or less removed from production. In our second study, we examined 17 BDE-congeners. BDE-47, a penta-BDE, was the congener present in the highest levels (median level: 83 ng per g wet weight of blubber). The levels of the other consistently detectable congeners were one (BDE-99, BDE-100, BDE-153, BDE-154&BB-153) or two (BDE-28,
BDE-49) orders of magnitude lower than BDE-47 levels, and overall values of these congeners correlated positively with BDE-47 values. The maximum level of BDE-209 was below detection limits (Chapter 4.2.). The first study had showed that levels of PBDE flame retardants did not increase from 1993 to 2002 (Chapter 4.1.), which may reflect the timely implementation of restrictions on the use of the most bioaccumulating compounds.

**Host genetics**

The point that had been highlighted by the 1988 PDV epidemic was that harbour seals exhibit strong population sub-structure, with significant genetic differences between populations that are easily within the reach of dispersing juveniles. Such structure has the potential to allow or even promote some level of inbreeding. Inbred individuals may be more susceptible to disease, and carry more and a greater diversity of parasites.

Two questions were addressed. The first was whether there was an association between the time of stranding in the PDV epidemic and heterozygosity, as had been previously observed in an outbreak of dolphin morbillivirus\(^8\). The second was whether there was an association between lungworm burden and heterozygosity. Lungworms are an important cause of death in between epidemics\(^7\), in particular in young seals\(^12\,\(^48\), and were fairly common co-infections in PDV-infected seals (in 11% of the 2002 sample, Chapter 3.1.). The harbour seal has two lungworm species: *Otostrongylus circumlitus*, a large species whose adults are easily visible in the principal airways, and *Parafilaroides gymnurus*, a smaller species found in the alveoli and smaller bronchioles. We used samples of 204 harbour seals that stranded along the Dutch coast between 1997 and 2003, and genotyped them for 27 microsatellites.

Crude analysis of the data showed no direct association between time of stranding during the PDV epidemic and heterozygosity, There was however an association between heterozygosity and worm burden, homozygosity predicting higher worm burdens, but only in young animals, where the worms have the greatest impact on fitness. Testing each locus separately, we found that a significant majority reveals a weak but similar trend for heterozygosity to be protective against high lungworm burden, suggesting a genome-wide effect, that is, inbreeding. This conclusion is supported by the fact that heterozygosity is correlated among markers in young animals but not in otherwise equivalent older ones. Taken as a whole, our results support the notion that homozygosity increases the susceptibility of young seals to lungworm infection (Chapter 5.1.).
Perspectives and conclusion

In the past decades PDV has twice caused major epidemics in harbour seals in the North Sea, before disappearing again from the area. No reliable historical records on PDV epidemics in harbour seals exist. Either PDV did not occur in the area, or alternatively the virus was endemic in the North Sea when harbour seal populations were larger, as it is thought to be now in seal populations in the Arctic and the western Atlantic\textsuperscript{19,22}. It may have disappeared with the decline and fragmentation of the population in the first half of the 20th century due to hunting, habitat loss, disturbance and pollution\textsuperscript{58,59}.

What caused the introductions of PDV infection in the North Sea is unknown, but it may be related to human activity causing change in marine mammal environment. One of the most important changes is probably related to intensive fishing practices, decreasing fish stocks both quantitatively and qualitatively\textsuperscript{3,36,55}. Another frequently cited cause is climate change induced by human activities\textsuperscript{5,40,42}. Host species from the Arctic and Atlantic environment have to adapt to these changes. Given the time-scale, adaptation is largely behavioral\textsuperscript{10}, for example following prey into new areas. This was assumed the cause of the harp seal movement into the North Sea in 1988\textsuperscript{17,46}.

At the population level, some species adapt more easily to a changing environment than others. Grey seals have recently joined harbour seals in the Dutch coastal waters\textsuperscript{60}, possibly becoming a competitor. Both species may feed on a large range of prey\textsuperscript{27,83}. Both harbour and grey seals make use of sandbanks to haul out, but grey seals also need land that is continuously dry to whelp and nurse and for pups to survive\textsuperscript{27}. However, grey seals are generally more robust and less susceptible to deleterious effects of infectious diseases, in particular phocine distemper\textsuperscript{20}. Even though the harbour seal population in the Netherlands has recovered in numbers since the 2002 epidemic (3595 harbour seals counted in the Wadden Sea in 2001\textsuperscript{80}, 4065 in 2006\textsuperscript{79}), PDV is likely to re-occur in North European waters and affect the harbour seal population again. Therefore PDV infection should definitely be accounted for when making management decisions concerning seal populations in European waters.

The threshold at which the harbour seal population will be large enough to allow PDV to persist is unknown. The size of the harbour and other seal populations in the North-western Atlantic may level off due to other factors such as food and habitat availability, by-catch and pollution\textsuperscript{36,69}, before being large enough to maintain PDV. Alternatively, anthropogenic influences as mentioned above may lead to more frequent introduction of PDV infection, with smaller more localized epidemics. Besides PDV, other morbillivirus infections are re-occurring in North European waters. For example, in 2006-2007, tens of long-finned pilot whales (\textit{Globicephala melas})\textsuperscript{23} and striped dolphins (\textit{Stenella coeruleoalba})\textsuperscript{57} stranded along the coast of the Spanish Mediterranean Sea and the Balearic Islands, DMV being the cause of the epidemic. In the summer
of 2007, Anholt was again the first site of a harbour seal die-off in Danish and Swedish waters. Most were pups and the macroscopic findings described were local increased consistency of the lungs with emphysema, but RT-PCR for PDV performed on lung tissue of 11 seals was negative and the cause remained undetermined. Recently, an assessment on the conservation status of the world’s mammals by the International Union for Conservation of Nature noted that threat levels are higher in marine than in terrestrial mammals, and knowledge poorer. These observations clearly illustrate the importance of continuous monitoring of marine mammal populations for infectious agents.

Our work has largely focused on insights into transmission events. Further understanding of this issue could be obtained if behavioural studies were done in which contact rates and types before and during an epidemic would be recorded. Also, during epidemics the stability of the virus in secretions on sandbanks and in mucus floating in the shallows should be addressed to determine the risk of infection from the environment.

Since the 1970s, we are more aware of the impact of anthropogenic environmental contaminants on human and ecosystem health. Measures are being taken to try to reduce the input of contaminants into the environment, and whenever possible to remove them from the environment. We found that important environmental contaminants did not significantly decrease in the North Sea in the 1990s. On the positive side, they did not increase either, and possibly measures have been implemented in a timely manner for more recently produced contaminants such as polybrominated-diphenyl-ethers (PBDEs). Efforts to reduce the input of anthropogenic environmental contaminants need to be pursued, amplified and taken to a global scale.

Taken together, this thesis describes multidisciplinary studies carried out in the Netherlands in response to a major PDV epidemic amongst harbour seals in north-western Europe in 2002, and compares the data with those obtained in response to a similar epidemic in the same area in 1988. PDV decimated half the harbour seal population of the Netherlands in 2002, without noticeably affecting the grey seal population. Compared to other North Sea locations, cumulative mortality in the Netherlands was on the high end of the range, and was similar to that of 1988. It is tempting to speculate that one of the reasons for this is that, like in 1988, environmental contaminants as PCBs were still at levels associated with immune suppression in harbour seals in 2002. The virus did not spread haphazardly through the population but followed a sex- and age-related pattern. Bordetella bronchiseptica infection was a frequent co-infection in PDV-infected seals both in 1988 and in 2002, while it was not isolated in between the PDV epidemics. Such PDV epidemics have not been documented before 1988, and the fact that they occur now may be partly due to human activities impacting on seal and fish populations and the marine environment in general. PDV is likely to re-occur in epidemic form, and because of its high pathogenicity for harbour seals, to substantially impact on their populations in the North
Sea. PDV should therefore be accounted for in North Sea harbour seal population management strategies.
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Nederlandse samenvatting

In april 1988 werd een ongewoon hoog aantal dode zeehonden gevonden op Anholt, een klein eiland in het Deense Kattegat. Dit was de start van de epidemie die in 1988 tussen 18.000 en 23.000 zeehonden in de Europese wateren zou doden, voornamelijk gewone zeehonden (*Phoca vitulina*). Onderzoek tijdens de epidemie toonde aan dat de sterfte veroorzaakt werd door een niet eerder gevonden morbillivirus, dat de naam ‘phocine distemper virus’ (PDV) of ‘zeehondenziektevirus’ kreeg.

In mei 2002 werd wederom een ongewoon hoog aantal dode zeehonden gevonden op Anholt. De diagnose PDV-infectie werd snel gesteld en bevestigd. De mogelijkheid dat dit zou leiden tot een epidemie in de Europese wateren lag voor de hand, omdat serologisch onderzoek had aangetoond dat het merendeel van de gewone zeehondenpopulatie waarschijnlijk geen antistoffen had tegen PDV. Beleidsmedewerkers in Nederland waren snel overtuigd van de noodzaak voorbereidingen te treffen om op de juiste manier op de epidemic te reageren en hem ook goed te documenteren. Nederland had toen vijf weken om zich voor te bereiden op de epidemic: het eerste geval van PDV-infectie in Nederlandse wateren werd vastgesteld op 16 juni 2002. In totaal zouden er in 2002 meer dan 22.500 zeehonden sterven in de Europese wateren.

De epidemic in 1988 leidde tot de publicatie van verschillende epidemiologische, pathologische en toxicologische studies, maar over de strandingen in Nederland werd weinig gepubliceerd. Het doel van dit proefschrift was om de PDV-epidemie in Nederland in 2002 vanuit deze verschillende disciplines te bestuderen, om daarmee zo mogelijk nieuwe inzichten te krijgen in de effecten van een PDV-uitbraak op individuele zeehonden en op de zeehondenpopulatie, en tevens in de gezondheidstoestand van het ecosysteem waarin deze zeezoogdieren leven.

Hieronder volgt eerst algemene informatie over zeehonden in Nederland en over PDV. Daarna worden de belangrijkste epidemiologische, pathologische en toxicologische bevindingen van de PDV-epidemie in 1988 samengevat.

**Achtergrondinformatie (Hoofdstuk 1)**

**Zeehonden**

Er leven twee soorten zeehonden in Nederland: de gewone zeehond en de grijze zeehond (*Halichoerus grypus*). Ze rusten in de Nederlandse Waddenzee en het Deltagebied op stranden of op zankbanken die bij dalend tij droogvallen en waarvandaan ze snel in diep water kunnen komen. Ze voeden zich met verschillende vissoorten en kreeftachtigen (*Crustacea*) in de wateren van deze gebieden en in de Noordzee.
Vrouwelijke gewone zeehonden zijn met drie of vier jaar geslachtsriep, mannelijke met vier tot vijf jaar, en ze kunnen tot zo’n 35 jaar oud worden. Geboorten komen in een bepaalde periode voor, die in Nederland van eind mei tot begin juli duurt. Pups zwemmen binnen enkele uren na de geboorte. De lactatieperiode duurt drie tot vier weken, waarna de pup abrupt gespeend wordt en paring tussen volwassenen plaats kan vinden. Implantatie van de bevruchte eicel wordt 2.5 maand uitgesteld. Gewone zeehonden ruien in de zomer, jaarlingen eerst, dan de overige onvolwassenen, dan volwassen vrouwtjes en het laatste volwassen mannetjes. Door het werpen en het ruien liggen gewone zeehonden in Nederland in de zomer vaker op land dan in de winter; in de winter wordt meer tijd doorgebracht in de Noordzee. In de zomer zijn ze ook tamelijk honkvast. Om deze twee redenen worden aantallen zeehonden geteld in de zomer. Geschat wordt dat het deel dat in de zomer op het land kan worden geteld ongeveer twee derde van de populatie bedraagt.


De gewone zeehond komt voor op het noordelijke halfrond, zowel in de Atlantische als in de Stille Oceaan. Van de vijf verschillende ondersoorten, komen er twee veelvuldig voor in de Noord Atlantische Oceaan: in het oosten P. vitulina vitulina (ongeveer 100.000 in 1985) en in het westen P. vitulina concolor (40.000-100.000 in de vroege negentiger jaren); de grens tussen beide populaties is onbekend. De geografische spreiding van de grijze zeehond beperkt zich tot de Atlantische Oceaan, met ongeveer 130.000 dieren in het oosten en meer dan 150.000 dieren in het westen.

Het virus: PDV

PDV behoort tot het genus Morbillivirus van de familie Paramyxoviridae. Het is een membraanvirus met een negatief enkelstrengs RNA. Het genoom bestaat uit zes genen, die acht eiwitten coderen: het nucleocapside eiwit (N), het fosfoproteïne (P), twee niet-structurele eiwitten C en V, het matrix eiwit (M), het fusie glycoproteïne (F), het hemagglutinine-glycoproteïne (H), en tenslotte het grote eiwit (L). M, H, en F zijn de membraaneiwitten van het virion; N, P en L, geassocieerd met het RNA, vormen het nucleocapside van het virion. H bindt aan cellulaire receptoren, en wekt de neutraliserende antilichaamrespons op. Het H-gen is het meest variabele deel van het genoom.

Er zijn tot nu toe acht morbillivirussen geïdentificeerd: het mazelen virus (MV), het runderpest virus (RPV), het ‘peste des petits-ruminants’ virus (PPRV), het hondenziekten virus (CDV), het dolfijn morbillivirus (DMV), het bruinvis morbillivirus (PMV), het griend morbillivirus (PWMV), en PDV. De laatste vijf infecteren zeezoogdieren, en de laatste vier zijn pas in de laatste drie decennia ontdekt. PDV is fylogenetisch het nauwste verwant aan CDV. PDV is in 1988 en in 2002 geïsoleerd uit natuurlijk geïnfecteerde gewone zeehonden, en in 1988 ook uit nertsen van een nertsenboerderij aan zee in Denemarken die vermoedelijk geïnfecteerd waren door contact met materiaal van gestorven zeehonden. Naast PDV infecties zijn bij verschillende zeehondensoorten ook infecties met CDV en met DMV waargenomen.

Bevindingen tijdens de PDV-epidemie van 1988


De bron van het virus bleef onbekend. Voorafgaand aan de epidemie hadden gewone zeehonden in de Noordzee nauwelijks antilichamen tegen morbillivirussen. In retrospectieve serologische studies werden wel antistoffen tegen morbillivirussen aangetoond in zeehondensoorten uit de Noord Atlantische Oceaan en Noordelijke IJszee, o.a. in zadelrobben (*Phoca groenlandica*),...
waarvan bekend was dat ze bij wijze van uitzondering in de Noordzee voorkwamen in de winter van 1987-1988. Opmerkelijk was eveneens de aanwezigheid van neutraliserende antistoffen tegen het PDV in één derde van de gewone zeehonden en meer dan twee derde van de grijze zeehonden bemonsterd langs de oostkust van de VS tussen 1980 en 1994.


Studies na 1988 toonden aan dat milieuverontreinigende stoffen in de zee, zoals polychloorbifenylen (PCB’s), het immuunsysteem van zeehonden negatief beïnvloedden. Deze stoffen hopen zich op in de zeehond, die, net als de mens, een van de laatste schakels is in de voedselketen.

Hogere gevoeligheid voor ziekten is wel in verband gebracht met lage genetische variabiliteit. Na 1988 werd d.m.v. genetisch onderzoek aangetoond dat er zes zeehondenpopulaties in Europese wateren zijn (Waddenzee; Engelse oostkust; West-Scandinavië; Oost Baltische Zee, Schotland-Ierland, IJsland) en dat de genetische variatie in de Waddenzee populatie lager is dan in de meeste andere populaties.

Epidemiologie (Hoofdstuk 2)

Beschrijving van de 2002 PDV-epidemie in Nederland

Hoofdstuk 2.1. beschrijft het verloop van de zeehondenstranding tijdens de 2002 PDV-epidemie in Nederland, alsmede de effecten van diergebonden (soort, geslachts- en leeftijdscategorie) en milieugerelateerde variabelen (locatie, wind, springtij, mate van ontbinding) op de dynamiek van de epidemicie. Ook werden een aantal epidemiologische kenmerken vergeleken, waaronder mortaliteit tussen de PDV-epidemieën van 1988 en 2002, gebruikmakend van zowel gepubliceerde informatie als van onze eigen gegevens.

Nederlandse samenvatting
tot de sectie plaatsvond. Tijdens sectie werden o.a. geslacht en lichaamslengte vastgesteld, op
grond waarvan zeehonden per geslacht ingedeeld konden worden in drie leeftijdsgroepen:
pups, onvolwassen zeehonden, en volwassen zeehonden. Gegevens over windrichting,
windsnelheid en springtij van het Koninklijk Nederlands Meteorologisch Instituut werden
eveneens verzameld.

De 2002 PDV-epidemie duurde in Nederland van 16 juni tot 29 november 2002. Er strandden 2284
zeehonden (2154 dood, 130 levend), waarvan 2279 gewone en 5 grijze. Van de 2284, strandden
er 2166 (95%) op de Waddenzee eilanden of het vasteland grenzend aan de Waddenzee. Er
werd sectie gedaan op 1315 carcassen, waarvan 1096 complete gegevens hadden en gestrand
waren op Vlieland, Terschelling, Ameland, Schiermonnikoog, Groningen, Friesland en Zeeland.
Bij deze 1096 gewone zeehonden werd het effect van de verschillende variabelen onderzocht.
De groep onvolwassen zeehonden strandde significant eerder dan die van volwassenen of
pups, en binnen elke leeftijdsgroep strandden mannelijke dieren over het algemeen eerder dan
vrouwelijke. Ook was er een effect van leeftijdsgroep op strandingslocatie: volwassen dieren en
pups waren proportioneel het meest talrijk op het vaste land van Groningen, en onvolwassen
zeehonden op Vlieland. Per kilometer strand was de dichtheid van strandingen het hoogste
op Schiermonnikoog. Alleen bij volwassenen verschilden de man-vrouw ratio's significant per
locatie. De mediane strandingsweek in Zeeland (week 39) was significant later dan die voor
alle Waddenzee locaties. Springtij had geen effect op het aantal dieren dat strandde, maar
windrichting wel: in perioden met zuidenwind strandden er minder dieren dan in perioden met
noordenwind. Het aantal dieren dat strandde in vergevorderde staat van ontbinding nam toe
naarmate de epidemic voortschreid, en was in de Waddenzee het hoogst op de kust van het
vaste land. De geschatte sterfte in 2002 was 54%, vrijwel identiek aan die in 1988 (53%).

Er strandden dus relatief meer gewone zeehonden dan grijze zeehonden, waarschijnlijk
omdat PDV-infectie in de gewone zeehond vaker een ernstig en dodelijke beloop heeft. De
verschillende strandingspatronen van de geslachts- en leeftijdsgroepen zouden verklaard
kunnen worden door geslachts- en leeftijds-gebonden verschillen in gedrag en indirect zouden
eventueel ook verschillen in gehalten aan milieuverontreinigende stoffen in het lichaam een
rol hebben kunnen spelen. Het was verbazingwekkend dat pups en volwassen vrouwtjes in juli
nauwelijks strandden. Dit zou kunnen komen doordat ze zich afzonderen tijdens het werpen
en in de zoogperiode, terwijl onvolwassen dieren in die tijd juist ruien en nauw contact met
elkaar hebben tijdens onderling spel. De geografische spreiding van strandingen kwam in
grote lijnen overeen met de normale verspreiding van zeehonden. Bijvoorbeeld de Eemsmond,
waar veel gestrandde pups waren gevonden, is ook een gebied waar veel pups geboren
worden. Het verschil in strandingspatroon tussen Zeeland en de Waddenzee zou het gevolg
cunnen zijn van een tragere verspreiding van PDV in deze relatief kleine en verspreid levende
zeehondenpopulatie. Wellicht de meest opvallende bevinding bij de vergelijking van de PDV-
epidemieen van 1988 en 2002 is dat de geschatte sterftepercentages in beide gevallen vrijwel
Nederlandse samenvatting

identiek waren.

**Factoren van invloed op de transmissie van morbillivirussen**

Bovengenoemde strandingspatronen waren aanleiding om een literatuurstudie uit te voeren naar de factoren die van invloed zijn op overdracht van morbillivirussen bij de mens en andere landzoogdieren. Het doel was om zo eveneens meer inzicht te krijgen in de overdracht van morbillivirussen bij zeezoogdieren, aannemend dat er parallelen zouden bestaan. Voor vijf uitgebreid bestudeerde morbillivirus-gastheer paren (mazelenvirus-mens; mazelenvirus-aap; runderpestvirus-rund; hondenziektevirus-hond; hondenziektevirus-fret) werden data samengevat voor acht factoren die bij overdracht een rol spelen: infectieroute, infectiviteit van het virus, gevoeligheid van de gastheer voor infectie en ziekte, virulentie van het virus, besmettelijkheid van de gastheer, uitscheidingsroute, stabilitiet van het virus in het milieu, en gedrag van de gastheer. Dit leidde tot de volgende conclusies.

Uitscheidings- en infectieroutes zijn van belang om na te gaan welk gedrag van de gastheer kan leiden tot een verhoogde kans op overdracht. Morbillivirussen worden hoofdzakelijk uitgescheiden via secreties uit neus, oog en mond en via urine, hoewel ook in uitgeademde lucht, uitwerpselen, sexuele vloeistoffen, bloed en huidkorsten infectieus virus aanwezig kan zijn. Infectie via de ademhalingswegen inclusief keelholte tijdens directe of indirecte contacten, bijvoorbeeld door druppels en aerosolen, vormt veelal de meest waarschijnlijke route. Dit suggeriert dat vooral interactief gedrag waarbij de nog niet geïnfecteerde gastheer met het hoofd contact heeft met lichaamsopeningen van de besmettelijke gastheer (druppeltjes secretie) geassocieerd is met een hoog risico voor overdracht. Bij gewone zeehonden doen zulke momenten zich vooral in de zomer voor, tijdens het spelen van onvolwassen dieren of bij aggressief gedrag tussen mannetjes (volwassen en bijna volwassen). Infectie kan ook voorkomen bij indirect contact tussen zeehonden, bijvoorbeeld door het ruiken aan secreta en uitwerpselen, of door inademen van aerosolen. Ook andere infectieroutes zijn mogelijk, zoals via de ogen, huidlesies, het maagdarmkanaal en het urogenitale systeem. Tezamen wijzen deze data er ook op dat infectie niet per se op het land hoeft plaats te vinden. De observatie dat morbillivirusinfecties van dolfijnen (DMV) en bruinvissen (PMV) efficiënt worden overgedragen wijst eveneens in deze richting. Gedragsstudies, zowel onder normale omstandigheden en gedurende morbillivirus-epidemieën, zouden hieromtrent meer inzicht kunnen geven.

Belangrijk voor het modelleren van morbillivirusinfecties zijn de lengte van de latente periode, en de duur en mate van infectiviteit in de infectieuze periode. De duur van de latente periode blijkt beïnvloed te worden door de infectiedosis. De besmettelijke periode duurt bij morbillivirusinfecties meestal slechts een paar dagen, maar kan al aanvangen wanneer de gastheer nog geen verschijnselen vertoont. Er is echter veel individuele variatie in duur en mate van besmettelijkheid. Er kunnen zogenaamde superspreaders voorkomen, die uitbraken
explosiever kunnen maken. Bij alle vijf bestudeerde morbillivirus-gastheerparen scheidt een aantall individuen het virus veel langer uit dan gemiddeld, veelal met tussenpozen, maar in het algemeen niet langer dan twee maanden. Het betreft hier veelal zwangere individuen die de ziekte hebben overleefd waarbij tevens de foetus is geïnfecteerd, en individuen met immuunstoornissen die al bestonden voor de infectie. Veelal ziet men dat zich bij deze individuen longontsteking of neurologische complicaties ontwikkelen. Herinfecties komen zeer zelden voor en de besmettelijkheid van zulke individuen is doorgaans laag.


Het is niet duidelijk waar PDV zichn tussen epidemieën ophoudt. De morbillivirussen van landzoogdieren hebben vaak één of twee reservoirgaasterhen, die gevoelig zijn voor morbillivirus infectie én ernstige, veelal dodelijke, ziekte. Op grond van dit criterium komt de grijze zeehond minder in aanmerking als reservoir dan de gewone zeehond. Een ander criterium om in aanmerking te komen als reservoir is populatiegrootte. Buiten meerdere reservoir-species, kan de populatiegrootte van de benodigde reservoir-gastheer afnemen als er minder virulente virusstammen circuleren, als de besmettelijkheid langer duurt, of als het virus buiten de gastheer langere tijd infectieus kan blijven. Morbillivirussen blijven buiten de gastheer doorgaans kort infectueus, maar bij bepaalde omgevingscondities, die virusstabiliteit positief beïnvloeden, kan infectieus virus lange tijd aanwezig blijven. Zo zijn temperatuur en luchtvochtigheid in het Noordpoolgebied gedurende de lange winter beide laag, en is er ook geen zonlicht. Hier is dus sprake van een combinatie van voorwaarden die de besmettelijkheid van excreta en karkassen in ruime mate kan verlengen.

**Modellen**

De kans op virusoverdracht is niet altijd dezelfde voor de gehele populatie, als gevolg van vele biologische en omgevingsfactoren zoals ruimtegebruik, genetische gevoeligheid, geslacht, en leeftijd. De mate van homogeniteit van de kans op overdracht tussen individuen, beïnvloedt de dynamiek van de epidemi.

Om het overdrachtspatroon van PDV tussen gewone zeehonden in de Nederlandse Waddenzee te bestuderen, deelden we allereerst de zeehonden in in drie leeftijdsgroepen op grond van geslacht en lichaamslengte: pup; onvolwassen zeehond; en, volwassen zeehond. Daarna werden drie verschillende “Susceptible-Infected-Removed” (SIR) modellen gemaakt in de vorm van
een WAIFW matrix. WAIFW staat voor “Who Acquires Infection From Whom”, ofwel “wie wordt geïnfecteerd door wie”. We maakten eerst een model waarin de kans op overdracht (transmissie factor beta) tussen alle individuen gelijk was (homogene ofwel volledig gemengde populatie); vervolgens een model waarbij de kans op overdracht binnen leeftijdsgroepen verschilde van de kans op overdracht tussen leeftijdsgroepen; en tenslotte een model waarin de kans op de overdracht binnen en tussen leeftijdsgroepen verschilde. Om de transmissiefactor beta te berekenen werd gebruik gemaakt van de incidentie van de strandingen van de verschillende leeftijdsgroepen.

Het derde model beschreef het verloop van de epidemic beter dan het tweede, en het tweede beter dan het eerste. Daaruit kan geconcludeerd worden dat het strandingspatroon beter verklaard kan worden als er rekening gehouden wordt met verschillen tussen leeftijdsgroepen. Overdracht van onvolwassen zeehonden naar volwassen zeehonden en vice versa was het hoogst, gevolgd door overdracht tussen onvolwassenen. Als controle werd de $R_0$ berekend als de dominante eigenwaarde van de ‘next generation matrix’ met de optimale combinaties van beta waardes. $R_0$ is het aantal secundaire gevallen dat ontstaat door besmetting door het eerste geval, in een volledig ontvankelijke populatie. Bij een $R_0 < 1$, spreidt de infectie niet; bij een $R_0 \geq 1$, wel. De berekende waarde van $R_0$ was 3.08, hetgeen goed overeenkwam met eerder berekende $R_0$ waarden voor PDV-infectie ten tijde van de epidemic van 1988. Veel van de transmissiefactoren konden meerdere waarden aannemen, en veel van de berekende waarden waren gecorreleerd. Gedragsstudies zouden meer inzicht kunnen geven in welke combinaties van beta-waardes het meest waarschijnlijk zijn.

Pathologie (Hoofdstuk 3)

Het grote aantal dieren dat voor sectie was verzameld tijdens de PDV-epidemie van 2002 bood een unieke gelegenheid om voor de beschrijving en analyse van de pathologie een quantitatieve benadering te gebruiken. Op monsters van 369 van de 1315 verzamelde karkassen konden diagnostische laboratoriumtests voor het aantonen van morbillivirusinfectie gedaan worden (reverse-transcriptase polymerase chain reaction, RT-PCR; immunohistochemistry, IHC; immunoglobulin M enzyme-linked immunosorbent assay IgM ELISA; immunoglobulin G enzyme-linked immunosorbent assay IgG ELISA), de overige karkassen verkeerden in een te gevorderde staat van ontbinding. Op grond van bevindingen bij de positief bevonden dieren (‘de PDV-gevallen’) werden vervolgens de lesies, de voedingstoestand en de co-infecties beschreven, alsmede de relaties tussen de testuitkomsten, lesies, leeftijd en co-infecties. Leeftijd werd bepaald door het tellen van het aantal cementlagen in een van de bovenste hoektanden, en voor analyse werden zeehonden ingedeeld in de drie genoemde leeftijdsgroepen (pups: <1-jaar oud, onvolwassenen: 1- of 2-jaar-oud, volwassenen: ≥ 3-jaar-oud). Een subset van 29 bevestigde PDV-gevallen werd gebruikt voor histologisch onderzoek, en een subset van 36 voor bacteriologisch onderzoek. Zeehondenkarkassen werden ook vóór de uitbraak systematisch verzameld en
onderzocht, waardoor het mogelijk was om de orgaangewichten, voedingstoestand en co-infecties van PDV-gevallen te vergelijken met die gevonden bij zeehonden die vóór de epidemicwaren gestorven door andere oorzaken dan PDV-infectie, zoals bijvangst, ongeval, mesenteriale torsie, long- of hart-worm infectie, of bacteriële infectie ('de niet-PDV-gevallen').

Van de 369 onderzochte zeehonden testten 232 positief voor morbillivirus infectie in een of meer laboratoriumtests (RT-PCR 47% positief; IHC, 54% positief; IgM ELISA, 37% positief; IgG ELISA, 27% positief). In theorie zouden dieren die slechts IgG positief waren, al ten tijde van de PDV-epidemie van 1988 geïnfecteerd kunnen zijn, en in 2002 aan een andere oorzaak dan PDV overleden zijn. In de praktijk bleek er maar één zeehond te zijn die alleén IgG positief was én ouder dan 13 jaar was. Er bleek geen negatief effect van invriezen en ontdooien op de uiteindelijke testresultaten te bestaan. Wel bleken karkassen waarvan het hersenweefsel positief werd bevonden met RT-PCR, in longweefsel vaak negatief te zijn, en vice versa. Dit suggereert dat een aantal neurologische gevallen geen detecteerbaar viraal RNA in hun longen hebben op het moment van overlijden, en wijst erop dat zowel longweefsel en het hersenweefsel getest moeten worden m.b.v. RT-PCR als PDV-infectie vermoed wordt. Morbillivirus antigeen werd het vaakst aangetroffen d.m.v. IHC in longen (bronchiale en alveolaire epitheelcellen, alveolaire macrofagen, en syncytia) en lever (galgangetheelcellen). De weefsels positief bevonden met RT-PCR waren niet altijd dezelfde als de weefsels die positief bleken met IHC. Er was geen correlatie tussen RT-PCR uitslagen en leeftijd, maar wel tussen IgG en leeftijd: het aantal morbillivirus IgG–positieve dieren nam significant toe met de leeftijd. Dit kan niet verklaard worden door verschillen aan blootstelling (PCR-resultaten geven hier geen aanwijzing voor), noch door reeds aanwezige IgG als gevolg van blootstelling in de vorige epidemic (nauwelijks gestrandde dieren ouder dan 13 jaar). Wel zou het verklaard kunnen worden door een minder vermogen van jonge zeehonden om specifieke IgG antistoffen aan te maken, bijvoorbeeld door een verhoogde gevoeligheid resulterend in een korter durend ziekteproces.

De belangrijkste lesies bij de 232 'PDV-gevallen' waren pneumonie (94% van de PDV-gevallen) en emphyseem (86% van de PDV-gevallen). Pneumonie was vaak ernstig (88% van de PDV-gevallen met pneumonie) en wijdverspreid (65% van de PDV-gevallen met pneumonie). Histologisch werd in meer dan de helft van de gevallen van pneumonie gekarakteriseerd als bronchopneumonie, en in de overige gevallen als broncho-interstitiële pneumonie, beiden meestal met bloedstuwing. Duidelijke, ronde, 3- tot 10-micrometer in diameter intracytoplasmatische en intranucleaire insluitlichaampjes waren soms zichtbaar in bronchiaal-, urineblaas-, pelvis-, of galgang-epitheelcellen. Bij de PDV-gevallen met emphyseem, was het emphyseem aanwezig in de longen (44%), mediast (91%), pericard (78%), retro-peritoneaal (52%), en/of onderhuids (68%), en beperkte zich zelden tot de longen (4%). Emphyseem ontwikkelt zich vermoedelijk omdat de longen van de zeehond sterk ontwikkelde interlobulaire septa hebben, en bij ernstige bronchopneumonie, lucht niet via de normale route van alveolus via bronchoelus en bronchus afgevoerd kan worden, en dus in de interlobulaire septa terecht komt,
en vandaaruit een uitweg zoekt en zich verder verspreid. Leeftijd had een significant effect op de verspreiding van emfyseem: extra-thoracale emfyseem kwam zelden voor bij pups en vaak voor bij volwassen dieren. Leeftijd had ook een significant effect op de ernst van de pneumonie en over de verspreiding van pneumonie: bij pups was longontsteking altijd ernstig en meestal uitgebreid. Een mogelijk verklaring hiervoor is een langere ziekteperiode bij oudere zeehonden. Een alternatieve verklaring zou kunnen zijn dat oude dieren langer duiken dan jonge dieren, omdat bij de laatste de duik- en zuurstofopslag-capaciteiten nog niet volledig ontwikkeld zijn. Voor het duiken worden longen luchtledig gemaakt, en als dat wordt belemmerd, zoals bij broncho-pneumonie, kan lucht achterblijven in alveoli, en dan door de druk in de septa terecht komen.

In 9% van de PDV-gevallen waren er ook andere lesies in de luchtwegen of in de borstholte gevonden, en in 7% van de gevallen duidelijke lesies in andere orgaansystemen. De laatste waren, in vergelijking met de rest, significant vaker IgG positief, en hadden minder vaak longontsteking en emfyseem. Dit alles suggereert dat deze dieren een langduriger en afwijkend ziekteverloop hadden in vergelijking met de meeste PDV-gevallen.

PDV-infectie had een significant positief effect op het gewicht van de longen, nier en bijnier van oudere dieren. Hoewel purulent bronchopneumonie zou kunnen leiden tot zwaardere longen, was er geen verband tussen matige of hoge aantal neutrofielen in longweefsel en het longgewicht. De meeste PDV-gevallen hadden lege magen (93%), of magen met alleen corpora aliena (4%). Een deel van de corpora aliena was sportvisapparatuur. Ook 'de niet-PDV-gevallen' die vóór de epidemie gestrand waren hadden meestal lege magen of magen met corpora aliena, behalve de zeehonden waarvan de dood veroorzaakt werd door bijvangst. De gemiddelde dikte van de speklaag bij PDV-gevallen (13.5 mm) was significant lager dan die van 'de niet-PDV-gevallen waarvan de dood geassocieerd werd met bijvangst (31.8 mm), en vergelijkbaar met die van 'niet-PDV-gevallen' met andere doodsoorzaken. Gezamenlijk zouden deze bevindingen kunnen wijzen op sterke dehydratie van dieren geïnfecteerd met PDV. In een toekomstige epidemie zou dit wellicht bevestigd kunnen worden door een ureum-stikstof bepaling uit te voeren op kamervocht van het oog.

Co-infecties waar systematisch naar gezocht werd waren infecties met phocine herpes virus 1, bacteriën, wormen en luizen. Van PDV-gevallen bleken 5% PCR positief voor phocine herpes virus 1 infectie. Bacteriële longontsteking kwam vaak voor, duidend op het immuunsuppressieve karakter van de PDV-infectie. We vonden 14 soorten of geslachten bacteriën, waarvan B. brochiseptica heeft meest voorkwam, nl. in tweederde van de onderzochte PDV-gevallen B. bronchiseptica werd altijd gekweekt uit longweefsel, en soms ook uit andere weefsels. B. bronchiseptica werd niet gevonden in de ‘niet-PDV-gevallen’. Macroscopisch was er minder aanwijzing voor bacteriële co-infecties dan microscopisch. Een verklaring zou kunnen zijn dat de bacteriële infecties in de longen veelal (per)acuut waren. Van de PDV-gevallen bleken
11% longwormen, 19% hartwormen, en 66% maagwormen te bevatten. De longwormen *Otostrongylus circumlitus* en *Parafilaroides gymnurus* kwamen vooral voor bij pups, en de hartworm *Acanthotheilonema spirocauda* vooral bij de onvolwassen dieren. De maagwormen *Pseudoterranova decipients* en *Contracaecum osculatum* werden gezien in alle leeftijdscategorieën, maar het hoogste aantal kwam voor bij de volwassen, en vooral de mannelijke zeehonden. Luizen waren aanwezig in 14% van de gevallen, vnl. bij onvolwassen dieren.

Samenvattend, wijzen onze resultaten uit dat voor PDV-diagnose d.m.v. RT-PCR zowel hersen- als longweefsels getest zouden moeten worden; dat leeftijds-gerelateerde verschillen in immunititeit en orgaan-ontwikkeling mogelijk het ziekteverloop en de ziekteduur beïnvloed; dat uitdroging een rol kan spelen bij het verloop en de uitkomst van zeehondenziekte en dat bacteriële co-infecties in de longen vaker voorkomen in PDV-gevallen dan het macroscopisch beeld suggereert. De evaluatie van deze resultaten illustreeert hoe kwantitatief pathologisch onderzoek bij massale epidemieën kan bijdragen aan het verkrijgen van beter inzicht in het algehele ziekteverloop van de oorzakelijke infectie.

**Toxicologie (Hoofdstuk 4)**

*De aanvankelijke afname in PCB- en DDT-gehalten in zeehonden houdt niet aan*

Sinds de jaren 1970, worden er beleidsmaatregelen genomen om te komen tot een schoner milieu. Dit leidde aanvankelijk tot een neerwaartse trend van veel organische chloorverbindingen in sediment en biota van de Noordzee, maar recente gegevens over trends van deze stoffen en nieuwe producten in top-predatoren zoals gewone zeehonden ontbreken grotendeels. Studies na 1988 toonden aan dat milieuverontreinigende stoffen zoals polychloorbifenylen (PCB’s) het functioneren van het immuunssysteem van zeehonden negatief kunnen beïnvloeden. Bij de aanvang van de 2002 epidemie werd gespeculeerd dat de zee mogelijk schoner zou zijn dan in 1988, en dus dat mortaliteit bij gewone zeehonden tijdens de PDV-epidemie wellicht lager zou zijn. Uiteindelijk bleek dat de cumulatieve mortaliteit in gewone zeehonden in de 2002 PDV-epidemie vergelijkbaar was met die van de 1988 epidemie, en bleef de vraag bestaan of verontreiniging hiertoe wellicht had bijgedragen.

Gehalten aan milieu-verontreinigende stoffen werden daarom bepaald in speklaag monsters van 45 onvolwassen gewone zeehonden gestrand tijdens de 2002 PDV-epidemie (*n* = 18) en de tien voorafgaande jaren (*n* = 27), en trends werden geanalyseerd. Waarden werden bepaald van polychloorbifenylen (PCB’s), dichloor-difenyl-trichloorethaan en metabolieten (DDTs), tris(4-chlorphenyl)methanol (TCPMOH), chloordaan verbindingen (CHLs), tris(4-chloorfenyl) methaan (TCPMe), heptachloorepoxide (HP-epox), hexachloorcyclohexaan isomeren (HCHs) en hexachloorbenzeen (HCB), en polygebromeerde difenylethers (PBDE’s). Onvolwassen dieren werden voor dit onderzoek gekozen omdat er dan weining invloed zou zijn van geslacht en
leeftijd op de gemeten gehalten.

De gemiddelde waarden (in vetgewicht) van de verschillende organische chloorverbindingen in speklaag waren in volgorde van hoog naar laag voor PCBs 28 μg/g, DDTs 3.0 μg/g, TCPMOH 0.80 μg/g, CHLs 290 ng/g, HP-Epox 61 ng/g, TCPMe 32 ng/g, HCHs 18 ng/g, en HCB 5.0 ng/g. PBDEs gehalten waren gemiddeld 380 ng/g, met vooral hoge BDE-47 waarden. In geen van de monsters was BDE-209 aantoonbaar.

Om de trend-analyse te doen, werd eerst voor elk van de organische chloorverbindingen en voor de PBDEs een lineair regressiemodel gemaakt met als stochastische variabele 'gehalte van de stof in speklaag' en als verklarende variabelen 'jaar van stranden' en 'speklaagdikte'. Alleen bij de organochloorpesticiden HCH en HCB was, na correctie voor speklaagdikte, een dalende trend waarnembaar in de periode 1993-2002. Bij alle andere organische chloorverbindingen, met inbegrip van PCBs en DDTs, en de PBDEs was geen trend waarnembaar in die periode. Voor alle stoffen behalve HCB was speklaagdikte een storende factor, significant en omgekeerd gecorreleerd met de gevonden gehalten. Zoals verwacht veranderden deze resultaten niet als 'geslacht', 'lengte', of hun interactie (als maat voor leeftijd) aan het model toegevoegd werden als verklarende variabelen. Alleen werd duidelijk dat vrouwelijke dieren bijna twee keer zo hoge TCPMOH en TCPMe gehalten hadden als mannelijke dieren. Vervolgens werden de gemeten PCB-, DDT- en HCH-gehalten gecombineerd met data van toxicologisch onderzoek bij onvolwassen gewone zeehonden uit de zuidelijke Noordzee in de zeventiger jaren. Hoewel een effect van de locatie niet kan worden uitgesloten, leek dit gegeven op een algemene daling van gehalten van PCBs, DDTs en HCH tussen de jaren 1970 en 1988. Daarna bleven HCH-gehalten dalen, maar verdwenen de neerwaartse trends voor DDT- en vooral PCB-gehalten grotendeels.

Tenslotte vergeleken we de gehalten aan PCBs, DDTs en HCB in gewone zeehonden die in 2002 kort voor of tijdens de PDV-epidemie stierven (n = 23), met gehalten uit een gepubliceerd experiment bij gewone zeehonden die gevoed waren met vis uit de sterk vervuilde Oostzee en die geassocieerd bleken met immuunsuppressie. De gemiddelde PCB-gehalten van de dieren uit 2002 waren in de zelfde orde van grootte als die van de dieren uit het experiment, terwijl gemiddelde DDT- en HCB-gehalten lager waren.

PBDEs

Polygebromeerde difenylethers (PBDE’s) zijn vlamvertragers geproduceerd en gebruikt sinds de vroege zeventiger jaren. PBDEs accumuleren in het milieu, en zonder beperkende maatregelen zou dit kunnen leiden tot nieuwe en langdurige milieuproblemen. Er waren drie commerciële producten in gebruik, pentaBDE, octaBDE en decaBDE, maar omdat schadelijke effecten voor de gezondheid vooral zijn aangetoond bij pentaBDE en octaBDE, zijn deze twee nu grotendeels verwijderd uit het productieproces. Het doel van deze studie was om een indruk te krijgen van de gehalten aan PBDEs in de gewone zeehond in de Nederlandse Waddenzee.

We bepaalden hiervoor de gehalten van 17 BDE-congeneren in speklaag monsters van 25 onvolwassen gewone zeehonden die tussen 1999 en 2002 strandden in de Nederlandse Waddenzee. BDE-47, een penta-BDE, kwam net als in de vorige studie in de hoogste gehalten voor (gemiddeld 122 ng/g vers gewicht van speklaag). De gehalten aan andere congeneren waren één (BDE-99, BDE-100, BDE-153, BDE-154 & BB-153) of twee (BDE-28, BDE-49) ordes van grootte kleiner dan de BDE-47 gehalten. De gehalten van al deze congeneren vertoonden een positieve correlatie met die van BDE-47-gehalten. BDE-209 was niet aantoonbaar. Zeehonden die gestrand waren in de westelijke Waddenzee hadden hogere BDE-47 gehalten dan dieren die gestrand waren in de oostelijke Waddenzee, hetgeen lijkt te correleren met de gehalten in vis. In de eerste studie was gebleken dat de niveaus van PBDE’s niet stegen van 1993 tot 2002. Wellicht zijn beperkende maatregelen voor pentaBDE en octaBDE tijdig genomen.

Zeehondengenetica en longworminfectie (Hoofdstuk 5)

De gewone zeehonden in Noordzee kunnen genetisch onderscheiden worden in meerdere sub-populaties. Deze populatieopbouw maakt een zekere mate van intellekt mogelijk of kan dit zelfs bevorderen. Ingeteelde individuen zijn vaak homozygoet. Over het algemeen zijn ingeteelde individuen meer vatbaar voor ziekten, en hebben ze een groter aantal en een grotere diversiteit aan parasieten, dan niet-ingeteelde individuen.

Twee vragen werden in het genetisch onderzoek van dit proefschrift centraal gesteld. De eerste was of er sprake was van een correlatie tussen het moment van de stranding in de PDV-epidemie en homozygotie, zoals eerder werd waargenomen bij een uitbraak van DMV-infectie in gestreepte dolfijnen (Stenella coeruleoalba). De tweede vraag was of er sprake was van een correlatie tussen longworminfectie en homozygotie. Longworminfecties zijn een frequente doodsoorzaak van jonge zeehonden. Er komen bij de gewone zeehond twee soorten longwormen voor: Otostrongylus circumlitus, een grote worm waarvan de volwassenen goed zichtbaar zijn in de bronchiën, en Parafilaroides gymnurus, een kleinere worm die zich bevindt in de alveoli en kleinere bronchioli.
We gebruikten 204 monsters van gewone zeehonden die tussen 1997 en 2003 langs de Nederlandse kust strandden, en we genotypeerden ze voor 27 microsatellieten (een kort stukje niet-coderend-DNA dat herhaald wordt). De zeehonden werden in twee longwormcategorieën ingedeeld: ‘geïnfecteerd’ als longwormen macroscopisch zichtbaar waren bij sectie; ‘niet-geïnfecteerd’, als dat niet het geval was. Verder werden ze ingedeeld in twee leeftijdsgroepen: ‘jong’ als zeehonden van vrouwelijk geslacht < 90 cm waren, of van mannelijk geslacht < 95 cm waren; de overige dieren waren ‘oud’. De maten gebruikt voor homozygotie waren ‘IR’ (internal relatedness) en ‘HL’ (heterozygosity weighted by locus).

Op grond van ruwe analyse van de data leek het dat er geen direct verband bestond tussen het tijdstip van stranden in de PDV-epidemie en homozygotie. Wel werd een positieve relatie tussen ‘homozygotie’ en ‘longworminfectie’ gevonden, maar dit alleen bij de jonge dieren. Dit heeft waarschijnlijk te maken met de levenscyclus van de longwormen. Deze infecteren gewone zeehonden waarschijnlijk vroeg in het leven, en al is de wijze van besmetting nog onbekend, het is waarschijnlijk dat de infectieuze larven zich in vis ontwikkelen en zeehonden oraal infecteren. Sommige jonge zeehonden zijn met meer longwormen geïnfecteerd dan anderen, soms met fatale gevolgen. De ontwikkeling van specifieke beschermende immunitie tegen longwormen is bij gewone zeehonden niet bewezen, maar zeehonden ontwikkelen wel antilichamen tegen longwormen, en longworminfecties bij oudere dieren zijn zeldzaam.

Om na te gaan of deze positieve relatie tussen ‘homozygotie’ en ‘longworminfectie’ bij de jonge dieren het gevolg was van een genoomwijde verandering in heterozygotie veroorzaakt door inteelt, of het gevolg van toevallige associaties tussen markers en de naburige genen onder gebalanceerde selectie, werden twee type analyses gedaan. De resultaten duidden in beide gevallen in de richting van een genoom-wijd effect, dat wil zeggen inteelt. Onder de volwassen dieren waren weinig ingeteelde dieren, onder de jonge dieren waren er meer. Deze resultaten passen dus bij de hypothese dat inteelt de vatbaarheid voor parasitaire ziekten verhoogt.

Samenvattende discussie (Hoofdstuk 6)

In dit Hoofdstuk worden de belangrijkste conclusies van dit multidisciplinaire onderzoek, dat werd uitgevoerd in aansluiting op de massale PDV-epidemie onder gewone zeehonden in Noordwest Europa in 2002, samengevat en in hun samenhang besproken. Gekozen werd voor een studie in het Nederlandse deel van het verspreidingsgebied van de gewone zeehond en de data werden waar mogelijk gerelateerd aan soortgelijke data verkregen na de PDV-epidemie van 1988 en aan data van de andere locaties in Europa. De belangrijkste conclusies waren dat PDV in 2002 de dood veroorzaakte van de helft van de gewone zeehonden in Nederland, zonder een duidelijk effect op de grijze zeehond te hebben. vergeleken met andere Noordzee-locaties, was het sterftepercentage in Nederland hoog en ongeveer gelijk aan dat van 1988. Het is mogelijk dat een van de redenen was dat, net als in 1988, milieuverontreinigende stoffen, zoals
PCBs, in zeehondenweefsels voorkwamen in gehalten die waarschijnlijk immunosuppressief zijn. Overdracht van het virus volgde een geslacht-en-leeftijdsgebonden patroon. *Bordetella bronchiseptica* infectie was een veel voorkomende co-infectie in de PDV-besmette zeehonden, zowel in 1988 en in 2002, terwijl deze bacterie in de tussenliggende periode niet geïsoleerd werd. PDV zal ook in de toekomst waarschijnlijk opnieuw in epidemische vorm optreden, en vanwege de hoge pathogeniteit voor gewone zeehonden, wederom leiden tot substantiële mortaliteit. Daarom dient terdege rekening gehouden te worden met PDV-infecties bij het beheer van de zeehondenpopulaties in de Noordzee.
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The author of this Ph.D. thesis was born in Kampala, Uganda, on the 23rd of November 1961. She went to English primary and French secondary schools in Africa. After studying biology for one year at the University of Amsterdam (1979-1980), she studied veterinary medicine at the Faculty of Veterinary Medicine of the University of Utrecht, from which she graduated in 1988. She obtained a M.Sc. degree in ‘Tropical Animal Health and Production’ at the Institut d’Elevage et de Médecine Vétérinaire des Pays Tropicaux in Paris (IEMVT, 1985-1986) and a M.Sc. degree in ‘Wild Animal Health’ at the Royal Veterinary College in London (1998-1999). She first worked fourteen years mostly with pastoralists in primary animal health care or rural development and natural resource projects in Africa: Karamoja, Uganda, 1988-1989; Batha Chad, 1989-1991; Nyala, Sudan, 1992; Dabola-Kouroussa-Dinguiraye, Guinea 1992-1994; Oudalan, Burkina Faso 1995-1998; Gaza-Inhambane, Mozambique 1998; Eastern Chad, 1999-2002. In 2002 she joined the Erasmus MC in Rotterdam to assist Prof. dr. A.D.M.E. Osterhaus and Prof. dr. T. Kuiken in setting up a Dutch Wildlife Health Centre (DWHC) together with the Faculty of Veterinary Medicine of the University of Utrecht. The 2002 phocine distemper virus epidemic was one of the wild animal health events in which the DWHC was involved. This event led in 2005 to the start of this Ph.D. thesis. Since March 2008, the author is working with the Egyptian Ministry of Agriculture and Land Reclamation to introduce participatory approaches to strengthen highly pathogenic avian influenza detection and response in Egypt.

Curriculum Vitae

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