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Detection of phocine herpesvirus 1 in seals from populations in different environments

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It always seems impossible until it is done
(Nelson Mandela)

für Papa Schwan
in tiefer Liebe und Dankbarkeit

Du warst noch bei uns, als ich diese Widmung zum ersten Mal schrieb. Ich war mir sicher, du würdest eines Tages lesen, woran ich arbeitete während ich Zeit mit dir hätte verbringen können. Du fehlst mir so sehr.

Abstract

Detection of phocine herpesvirus 1 in seals from populations in different environments

Phocine herpesvirus 1 (PhHV-1) is a large double stranded DNA virus belonging to the family *Herpesviridae*, subfamily A*lphaherpesvirinae*, genus *Varicellovirus*. It was first isolated and partially characterized during a disease outbreak in young harbor seals (*Phoca vitulina*) in the North Sea. The disease is frequently seen in seal pups worldwide and is associated with high morbidity and mortality. PhHV-1 isolates from seals of the Atlantic / European waters and isolates from seals ranging in the Pacific Ocean differ genetically and in manifested post-mortem findings in diseased animals. Whilst disease in European seals is associated with pneumonia and focal hepatitis, the most striking feature found in seals from Pacific waters is adrenal necrosis. Few studies have addressed seroprevalences of PhHV-1 in different species of free ranging seals, mainly in the Northern hemisphere. Particularly seals in captivity often develop bilateral ocular disease of unknown etiology. These symptoms are also found in free ranging populations but with lower frequency. It is hypothesized that PhHV-1, similar to feline herpesvirus type 1 (FeHV-1) in terrestrial carnivores, may contribute to the etiology of ocular disease during reactivation events. A PhHV-1-specific quantitative real-time PCR (qPCR) assay was established based on an alignment of known PhHV-1 glycoprotein B sequences. The assay was used to detect viral DNA in ocular, nasal and genital swabs from free ranging harbor seals, harp seals (*Phoca groenlandica*) and hooded seals (*Crystophora crystata*) of the Arctic and Antarctic fur seals (*Arctocephalus gazella)*, Weddell seals (*Leptonychotes weddellii*), and Southern elephant seals (*Mirounga leonida)* of the Antarctic. The finding of PhHV-1 DNA in genital swabs was unexpected and raises the question whether the virus may, similar to bovine or equine herpesviruses, contribute to reproductive problems. An indirect PhHV-1 ELISA was developed and harbor seals of the Arctic as well as Antarctic fur seals, Weddell seals, crabeater seals (*Lobodon carcinophaga)*, Ross seals (*Ommatophoca rossii*) and Southern elephant seals of the Antarctic were tested serologically. Harbor seal sera were available from a period spanning 12 years, enabling long-term monitoring of PhHV-1 in this population. Antibodies against PhHV-1 were detected in Ross seals and Southern elephant fur seals for the first time, which demonstrates that these species are susceptible to PhHV-1.

Kurzzusammenfassung

Nachweis von phocinem Herpesvirus 1 in Robben in unterschiedlichen Populationsgebieten

Phocines Herpesvirus 1 (PhHV-1) gehört als großes, doppelsträngiges DNA Virus der Familie *Herpesviridae*, Subfamilie *Herpesvirinae*, Genus *Varicellovirus* an. Erstmals isoliert und partiell charakterisiert wurde das Virus bei erkrankten jungen Seehunden (*Phoca vitulina*) in der Nordsee. PhHV-1 ist weltweit verbreitet und Erkankungen sind assoziiert mit hoher Morbidität und Mortalität. Virusisolate von Tieren im Antlantik / in europäischen Gewässern differieren genetisch von Isolaten von Robben aus dem Pazifik und auch postmortale Befunde bei erkrankten Tieren sind unterschiedlicher Ausprägung. Während Pneumonie und hepatische Nekrosen das pathologische Bild in mit europäischen Isolaten infizierten Robben dominieren, sind adrenale Nekrosen ein charakteristischer Hauptbefund in pazifischen Robbenarten. Nur wenige Studien berichten bisher über PhHV-1 Seroprävalenzen in wild lebenden Robben, die meisten davon über Tiere der nördlichen Hemisphäre. Insbesondere bei Tieren in Zoos und Aquarien werden häufig bilaterale Augenerkrankungen unbekannter Ätiologie diagnostiziert. Die Symptomatik ist auch, jedoch seltener, bei wild lebenden Tieren bekannt. Da die Reaktivierung von felinem Herpesvirus 1 (FeHV-1) in Katzen eine sehr ähnliche Symptomatik verursacht, wurde die Hypothese aufgestellt, dass PhHV-1 an der Ätiologie von Augenerkrankungen in Robben beteiligt ist. Ein PhHV-1 spezifischer quantitative-Echtzeit-PCR (qPCR) Assay, basierend auf Alignments bekannter PhHV-1 Glycoprotein B Sequenzen, wurde entwickelt, um Augen-, Nasen- und Genitaltupferproben frei lebender Seehunde, Sattelrobben (*Phoca groenlandica*) und Klappmützenrobben (*Crystophora crystata*) aus der Arktis und Seebären (*Arctocephalus gazella)*, Weddellrobben (*Leptonychotes weddellii*), und See-Elefanten (*Mirounga leonida)* aus der Antarktis auf PhHV-1 DNA zu untersuchen. Die erstmalige Detektion von PhHV-1 DNA in Genitaltupferproben wirft die Frage auf, inwiefern das Virus, ähnlich bovinen oder equinen Herpesviren, in Reproduktionsprobleme involviert sein könnte. Ein indirekter PhHV-1 ELISA wurde entwickelt und Seehunde der Arktis sowie Seebären, Weddellrobbe, Krabbenfresser (*Lobodon carcinophaga*), Rossrobben (*Ommatophoca rossii*) und See-Elefanten der Antarktis wurden serologisch getestet. Serumproben von Seehunden über einen Zeitraum von 12 Jahren ermöglichten die Beobachtung dieser Population hinsichtlich PhHV-1 Seroprävalenzen über längere Zeit. Erstmals konnten Antikörper auch in Rossrobben und See-Elefanten festgestellt werden, was indiziert, dass diese Spezies empfänglich für PhHV-1 sind.

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List of Abbreviations

aa	amino acid
bp	base pair
CDV	canine distemper virus
CHV	canine herpesvirus
CPE	cytopathogenic/cytopathic effect
CRFK	Crandell feline kidney cells
C_T	cycle threshold
CV	coefficient of variation
Da	Dalton
DeHV	delphinid herpesvirus
DNA, RNA	deoxyribonucleid acid, ribonucleid acid
ELISA	enzyme-linked immunosorbent assay
EHV	equine herpesvirus
FHV	Feline herpesvirus
PCR, qPCR	polymerase chain reaction, quantitative real-time PCR
gB (D,E,I)	glycoprotein B, D, E, I
HSV-1	herpes simplex virus
ICTV	International committee for the taxonomy of viruses
IR	internal repeat
ISCOM	immune stimulating complex
kbp	kilobasepair
KoHV	kogid herpes
LAT	latency associated transcripts
MOI	multiplicity of infection
NCBI	National Center for Biotechnology Information
NARA	Norwegian Animal Research Authority
ORF	open reading frame
OtHV	otariid herpesvirus
PDV	Phocine distempervirus
PhHV	Phocine herpesvirus
Pol	DNA dependent DNA polymerase
RT	room temperature
SD	standard deviation

SL	standard length
SNP	single nucleotide polymorphism
SNT	serum neutralization test
TR	terminal repeat
TrHV	trichechid herpesvirus
U_L	unique long
U_S	unique short
VN	virus neutralization

Chapter I - INTRODUCTION

1. Herpesviruses

General features of herpesviruses and current knowledge about PhHV-1 are presented in the following paragraphs. For general herpesvirus properties, features of the genus *Varicellovirus* and particularly FeHV-1 are emphasized. FeHV-1 is genetically and symptomatically closely related to PhHV-1 and has been, due to its relevance in domestic cats, more intensely studied than the marine mammal virus.

The family *Herpesviridae* forms, together with the families *Alloherpesviridae* (herpesviruses in fish and amphibians) and *Malacoherpesviridae* (herpesviruses in bivalves) the order *Herpesvirales*. Taxonomic classifications are given according to the International Committee on Taxonomy of Viruses, ICTV (Büchen-Osmond, 2002). Herpesviruses are globally distributed and highly disseminated in nature, causing disease in a wide range of vertebrates including mammals, reptiles and birds (Davison, 1997). New herpesviruses are still discovered frequently and more than 200 species, causing disease in humans and various animals, are identified to date (Pellett, 2006). Some of which have a wide, others a rather narrow host range.

Viruses were originally classified based on morphologic criteria of the virion and on biological properties. A typical herpesvirus virion consists of the linear, unsegmented, double stranded, 100-300 kbp long DNA genome within the core, the icosahedral capsid (approximately 125 nm in diameter), the amorphous tegument and the envelope with glycoprotein spikes (Pellett, 2006). A schematic of a herpesvirus virion is illustrated in figure I-1. Despite great variability in pathology and biology of infection, herpesviruses share one main common biological feature: the ability to establish latency after primary infection (Davison, 1997).

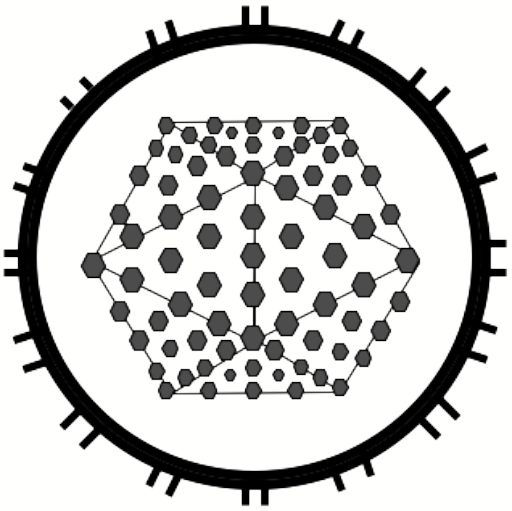


Figure I-1: Herpesvirus morphology Schematic of a herpesvirion: envelope with glycoprotein spike projections enclosing capsomeres, which are forming the icosahedral capsid with typical 5:3:2 rotational symmetry

1.1. Common features of herpesviruses

Despite their common morphological characteristics, three biological properties are shared by members of the family *Herpesviridae*: a) a large number of proteins needed for nucleic acid metabolism, synthesis and the processing of viral proteins is encoded in the viral genome; b) DNA replication and assembly of progeny capsids take place in the nucleus, further processing of virions occurs in the cytoplasm; c) the virus has the unique ability to remain latent in the host after primary infection (Pellett, 2006). The viral genome is kept silent in form of an episome or, less often, integrated into host cell chromosomes of specific cells whilst only few virus genes are expressed and no infectious progeny virus is produced. The virus remains the ability to return to productive, lytic replication and reactivation occurs upon certain triggers.

Nomenclature and classification

The name herpesvirus derived from the Greek word herpes = “to creep”, describing disease symptoms, particularly skin conditions associated with the virus (Roizman, 1982). Virus species were designated descriptively after the clinical picture they caused (e.g. pseudorabies), after their discoverers (e.g. Epstein-Barr virus), after pathological findings (e.g. cytomegalovirus) or after their hosts (e.g. herpesvirus hominis). Many of those common names are still being used, despite their disaccord with today’s rules for the classification of viruses established by the ICTV.

Before nucleotide and amino acid sequences were known, members belonging to the family *Herpesviridae* were initially classified based on their biological properties such as host range, duration of reproductive cycle, cytopathology and characteristics of latent infection (Pellett, 2006). They were clustered into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. This classification was supported by nucleotide sequence information later on. Today, herpesviruses are further systematized into genera, considering DNA sequence homology, similarities in genome arrangement and similarity of viral proteins.

The subfamily *Alphaherpesvirinae* comprises the genera *Iltovirus*, *Mardivirus*, *Simplexvirus*, *Varicellovirus* and various unassigned virus species (ICTV). Most of them are replicating rapidly, usually associated with destruction of susceptible cells, causing clear cytopathic effects (CPE) *in vitro* (Roizmann et al., 1992). Some viruses, particularly members of the genus *Mardivirus*, are slowly cytopathic (Barthold, 2011). Alphaherpesviruses have a variable, wide to narrow host range and many members cause vesicular epithelial lesions. Sensory ganglia are predominantly but not exclusively targeted for latency, e.g. EHV-1 establishes latency also in lymphocytes (Welch et al., 1992).

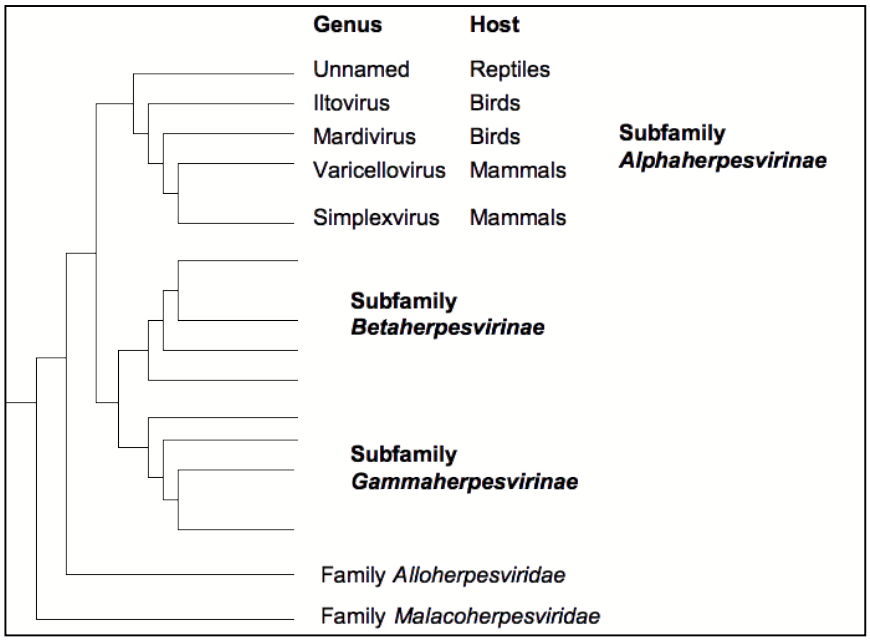
The subfamily *Betaherpesvirinae* incorporates the genera *Cytomegalovirus*, *Muromegalosvirus*, *Roseolovirus* and few unassigned species (ICTV). Betaherpesviruses are characterized by a long reproductive cycle and hence slow *in vitro* growth (often associated with cytomegalia) (Barthold, 2011). Many species remain cell associated and latency is established in various tissues and organs such as secretory glands or kidneys. The host range is mostly narrow to highly restricted.

The subfamily *Gammaherpesvirinae* comprises the genera *Lymphocryptovirus*, *Rhadinovirus* and one unassigned species (ICTV). Members are also known as lymphoproliferative herpesviruses, predominantly targeting T or B lymphocytes and establishing latency in lymphoid tissues (Mahy, 2001). Some members are linked to oncogenic transformation and the host range is narrow (Barthold, 2011).

Major herpesvirus phylogenetic relationships and genera with their respective host species belonging to the subfamily *Alphaherpesvirinae* are illustrated in figure I-2.

Figure I-2: Herpesvirus - phylogenetic relationships and genera

Hosts within the subfamily *Alphaherpesvirinae*, family *Herpesvirinae*
Adapted from Fig. 66.2 in Field's Virology p. 2481 (Pellett, 2006)



Structure

With the introduction of electron microscopy, the identification of morphological features of viruses became possible and a detailed description of herpes simplex virus, analyzing negative stain electron microscopy pictures, was published in 1960 (Wildy et al., 1960). Herpesvirus virions are 120 – 260 nm in diameter. The large variation is caused by variable

dimensions of the tegument (Barthold, 2011; Pellett, 2006). Virions are made of a large number of virally encoded proteins as well as host-derived proteins and consist of three main structural elements: the icosahedral nucleocapsid, the tegument and the envelope.

Describing the components in more detail from the inside out, virions contain the viral DNA to start with. The nucleid acid is wrapped toroidally around a protein spindle (Furlong et al., 1972). The capsid consists of 162 capsomers with 150 hexons and 12 pentons, originally described as elongated hollow prisms with polygonal cross-sections (Wildy et al., 1960), illustrated in figure I-1. Each capsomer is formed by four essential capsid proteins of which the major capsid protein is present in six copies per hexon, five per penton and hence a total number of 960 copies per capsid (Pellett, 2006). Roizman and Furlong first described the virion component between capsid and envelope, the tegument (Roizman, 1974). The amorphous structure delivers fully synthesized proteins into newly infected cells, which are able to immediately modulate the host environment in favor of the virus (Pellett, 2006). The lipoprotein envelope is derived from cellular membranes (Darlington and Moss, 1968). Protrusions within the envelope are glycoproteins of which number and relative amount on the surface vary between species (Pellett, 2006).

Genome

The herpesvirus genome is linear and double stranded. Circularization occurs immediately after the DNA is released from the capsid into the nucleus of the infected cell (Wadsworth et al., 1976) and DNA in the form of genome concatemers can be found after rolling-circle replication (Jacob et al., 1979). The degree of variation regarding genome length, composition and organization is high within the family *Herpesviridae* (Barthold, 2011; Murphy, 1999). Genome lengths vary between 100 and 300 kbp, encoding 70 - 165 genes (Norkin, 2010). Differences in genome length are associated with variable copy numbers of terminal and internal repeated sequences (Roizman, 1979). The base composition of herpesviral DNA across the genome varies substantially more than that of eukaryotic DNA, with extensive variations of the GC content as large as 30 - 40% over one genome (Roizman, 1980). The complex fashion of sequence arrangements is characterized by unique and repeated sequence elements (Pellett, 2006). Alphaherpesvirus genomes contain two unique regions, intermitted and flanked by reiterated DNA sequences (Barthold, 2011). Given as an example for the complexity of herpesviral genomes, a classical arrangement scheme of varicelloviruses with unique long (U_L), inverted repeats (IR), unique short (U_S) and terminal repeats (TR) is schematically illustrated in figure I-3.

Figure I-3: Genome organization of herpesviruses genus *Varicellovirus*

modified from Fields Virology p. 2490 figure 66.4
The two unique regions, U_l = unique long and U_S = unique short, are divided by inverted repeats (IR), a region of terminal repeats (TR) is flanking U_S at the right end of the genome

Genes are overlapping frequently and promoter regions of genes are often located within the coding sequence of the gene located upstream (Pellett, 2006). Poly(A) sites can be shared but most genes have their own promoter and termination site (Norkin, 2010). Multiple functions of herpesvirus gene products are common and the actual function is determined by posttranslational modifications. A large number of genes is dispensable for growth in cell culture but essential for viral replication or reactivation *in vivo*. Proteins are often classified according to their mode of expression into immediate early genes (required even before the onset of viral protein synthesis for the expression of other viral gene products), early genes (independent of viral DNA synthesis), leaky late genes (enhanced through viral DNA synthesis) and true late genes (dependent on viral DNA synthesis). A number of essential genes involved in replication and structure, so called core genes, are conserved across the subfamilies. Other genes are conserved at the subfamily level or optional and hence non-essential.

Herpesvirus infectious cycle

A main characteristic of herpesviruses is their ability to remain latent in the host organism after a first infection (Modrow, 2003; Norkin, 2009). During latency, the production of infectious particles is abandoned until the virus is reactivated and reenters the lytic replication cycle. Infected hosts therefore become lifelong reservoirs of the virus.

The infection of a cell with a herpesvirus begins with the interaction of viral glycoproteins and host cell membrane proteins, leading to the fusion of viral and cellular membranes (Modrow, 2003). First binding occurs mainly as binding to glycosaminoglycans, whilst entry is triggered by the interaction of glycoproteins with specific entry receptors (Norkin, 2010). Following membrane fusion, viral nucleocapsids enter the cytoplasm and are transported via microtubules to the nucleus. They dock at nuclear pore complexes and the viral genome is released into the nucleoplasma, where it circularizes (Modrow, 2003). Viral proteins with nuclear localization/transport signaling domains are transported into the nucleus as well. Expression of the viral genome begins with proteins encoded by immediate early genes, which act as regulators for further transcription. The replication of virus DNA during the lytic

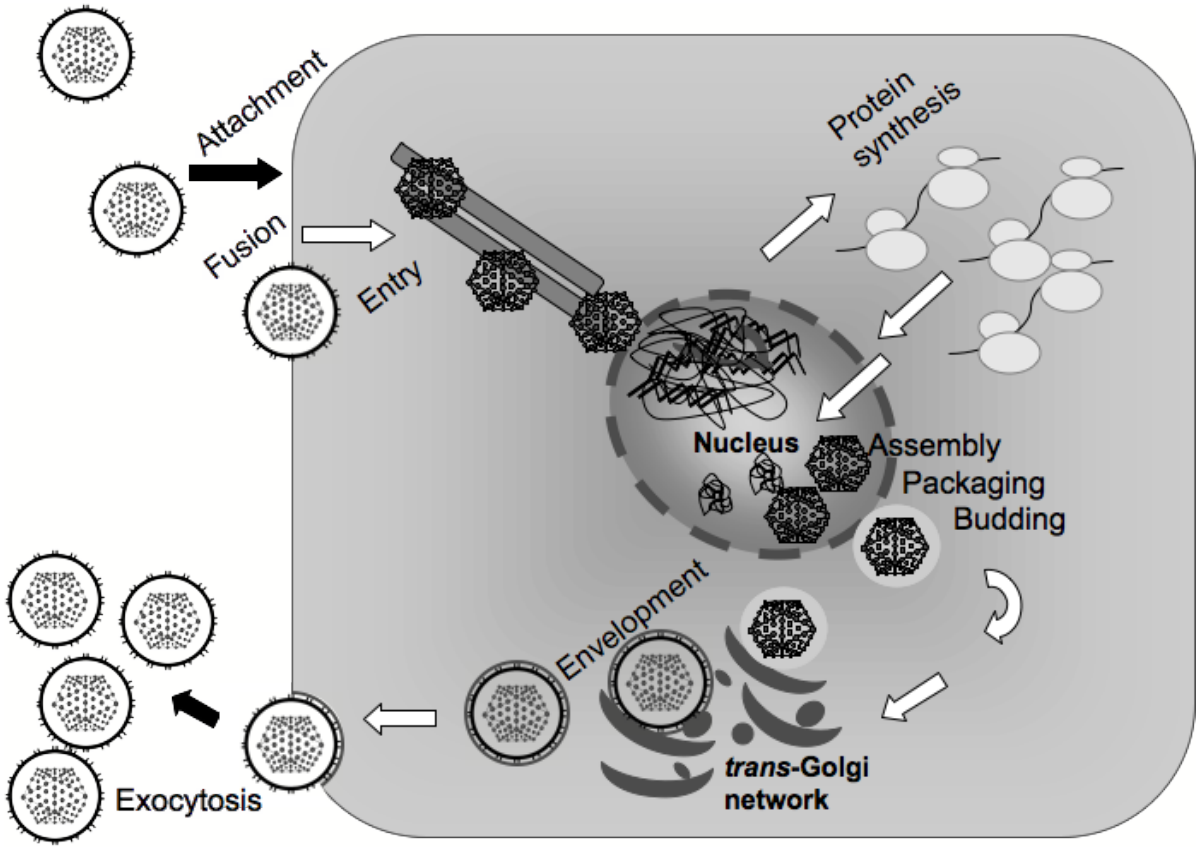
phase is implemented according to the *rolling circle* principle, generating progeny genomes in concatemeric form. Attachment of the virally encoded polymerase-helicase-primase complex is mediated by a virus protein, which is binding the origin of replication. One DNA strand is cut and synthesis is carried out along the circular template strand. Concatemers are further cleaved into unit-legth genome copies upon packaging.

During DNA replication, late viral proteins are synthesized in the cytoplasm and capsid and tegument components are transported to the nucleus, where capsid precursors are assembled (Norkin, 2010). Single genome copies resolved from concatemers are packaged into empty procapsids and mature capsids leave the nucleus via budding. Capsids are further processed to infectious particles at the *trans*-Golgi network, where they obtain their final envelope. Progeny virions are transported to the cell surface via Golgi vesicles and released via exocytosis or cytolysis. Alternatively, infectious virions can be transmitted via cell-to-cell contact and cell fusion.

The lytic replication cycle of herpesviruses is illustrated in Figure I-4, the latent infectious cycle is emphasized in a separate paragraph below.

Figure I-4: Schematic illustration of the herpesvirus lytic replication cycle

Attachment of particles, fusion, release of nucleocapsids into the cytoplasm, transport along microtubules to the nucleus, docking to nuclear core complexes, intranuclear replication of viral DNA, protein synthesis in the cytoplasm, capsid assembly and packaging of the viral genome, budding from the nuclear membrane, envelopment at the *trans*-Golgi network, exocytosis and release of virions



Latency and reactivation in herpesviruses

Latency is a mechanism, by which herpesviruses escape the immune response and are able to persist over long periods of time in the absence of disease before reactivation of the lytic cycle (Norkin, 2010). Quiescence in specific host cells is a herpesvirus-specific feature, which can be found in various characteristic forms. Three factors are involved in the regulation and maintenance of latency: the virus itself, the target cell and the host immune system as it was shown for HSV-1 (Divito et al., 2006). During latent infections, most viral genes are not or inefficiently expressed, the pathogen cannot or can only be insufficiently recognized by the immune system and the viral genome persists in host cells (Flint, 2003). Maintenance of the genome can occur as a non-replicating chromosome in non-dividing cells such as neurons, as an autonomous self-replicating episome in dividing cells as is the case for Epstein-Barr virus infected B-cells, or integrated into the host chromosome (Kaufer et al.). The copy number of latent DNA can vary and was reported to range from one to 1000 genomes (Sawtell, 1997). How the initiation and completion of the lytic replication cycle is repressed and how latency is established is regulated in a virus-specific and complex manner. Transcription and gene expression are limited to latency associated transcripts (LAT). Production of infectious virus at later time points requires reactivation. The change from latent stages back to lytic infection may be spontaneous or triggered by trauma, stress or other conditions that may endanger the continuation of latency from the host side. (Sekizawa et al., 1980)

Many alphaherpesviruses develop neuronal latency in sensory ganglia (Modrow, 2003). To reach their target cells, viruses are transported in a retrograde manner along axons (Bearer et al., 2000). Other alphaherpesviruses establish latency in peripheral blood leucocytes (Cantello et al., 1994; Welch et al., 1992). For FeHV-1, the trigeminal ganglion is a known site of latency, identified by the detection of LATs (Gaskell et al., 1985). From trigeminal ganglia, viruses are transported anterogradly to the periphery where they can establish recurrent lesions upon reactivation (Stiles, 2003).

Pathogenesis

Herpesviruses are versatile pathogens, exhibiting divergent outcomes, symptomatology, infection characteristics and pathogenesis (Barthold, 2011).

Transmission mostly occurs via mucosal contact or aerosol (Barthold, 2011; Murphy, 1999). Alphaherpesvirus infections often cause localized lesions on skin, mucosae and/or in the respiratory and genital tract. Newborns or immunocompromised animals may develop generalized infections characterized by foci of necrosis in various organs and tissues. Transplacentar transport of virus via cell-associated viremia during pregnancy can lead to vertical transmission and can cause abortions. Virus invades sensory ganglions or alternative

sites of latency, where the quiescent genome remains silent despite the expression of LATs. Reactivation is usually associated with stressful and/or immunosuppressive conditions and leads to further shedding of virus and transmission. Infections with beta- and gammaherpesviruses are often but not exclusively without clinical symptoms.

In the case of FeHV-1, virus is transmitted directly to other individuals via oro-nasal and conjunctival route from cats shedding virus in ocular, nasal and oral secretions (Gaskell et al., 2007). The primary site of virus replication are epithelial tissues, leading to typical signs of upper respiratory disease with high morbidity (Stiles, 2003). Infections are normally restricted to the upper respiratory tract. Viremia has only been reported occasionally (Hoover et al., 1970). Kittens infected primarily with FeHV-1 develop more general signs of disease and rarely develop viral or bacterial pneumonia resulting in death (Stiles, 2003). The establishment of latency in trigeminal ganglia was substantiated by the detection of LATs (Ohmura et al., 1993). The detection of viral DNA but not LATs in the cornea and other ocular neurological sites impedes the distinction of low grade viral reactivation from latency (Gaskell et al., 2007). Recurrences of FeHV-1, mainly occurring in older cats, are mostly associated with signs of mild upper respiratory disease and corneal ulcerations (Stiles, 2003).

1.2. Herpesviruses in marine mammals

Phylogenetic branching in herpesviruses mirrors those seen in their host species, which leads to the conclusion that herpesviruses have co-emerged and co-diverged with their host species (Pellett, 2006). With about 5500 mammal species known, the finding of many more herpesvirus species can be expected (Maness et al., 2011). A comprehensive summary of herpesviruses in marine mammals identified to date and appendant phylogenetic analyses was published recently (Maness et al., 2011). Established viral DNA polymerase sequences were compared with four novel sequences from dolphins, seals and sea lions. No complete marine mammal herpesvirus genome has been sequenced so far, but DNA polymerase nucleic acid sequences have been published for following species: Delphinid herpesvirus (DeHV) 1-9 isolated from Bottlenose dolphins (*Tursiops truncatus*; DeHV-1 - 5, 7, 8), a Risso's dolphin (*Grampus griseus*; DeHV-6) and an orca (*Orcinus orca*; DeHV-9); Kogiid herpesvirus (KoHV-1) in a dwarf sperm whale (*Kogia sima*); Otariid herpesvirus (OtHV) 1 and 2 from California sea lions (*Zalophus californianus*); Phocid Herpesvirus (PhHV) 1-5 isolated from harbor seals (*Phoca vitulina*; PhHV-1,2 and 5), from Hawaiian monk seals (*Monachus schauinslandi*; PhHV-3) and from a Northern elephant seal (*Mirounga angustirostris*; PhHV-4); Trichechid Herpesvirus (TrHV-1) in Florida manatees (*Trichechus manatus latirostris*;

TrHV-1); and Ziphid herpesvirus (ZiHV-1) in a Blainville's beaked whale (*Mesoplodon densirostris*; ZiHV-1). In NCBI GenBank, gB and gD sequences are available only for PhHV-1 (table I-1).

Osterhaus et al. reported the first herpesvirus in a marine mammal in 1985, the alphaherpesvirus PhHV-1 in harbor seals (Osterhaus et al., 1985), which was found later in a grey seal (*Halichoerus grypus*) as well (Maness et al., 2011). In 1994, a novel gammaherpesvirus was isolated from harbor seal leukocytes, PhHV-2 (Lebich et al., 1994). Other pinnipeds, in which herpesviruses were found, are Hawaiian monk seal, California sea lion and Northern elephant seal (Goldstein, 2006a, b).

Within the order *Cetacea* (Cetaceans: dolphins, porpoises and whales), herpesviruses were found in beluga whales (*Delphinapterus leucas*) (Barr et al., 1989; Martineau et al., 1988), harbor porpoises (*Phocoena phocoena*) (Kennedy, 1992), dusky dolphins (*Lagenorhynchus obscures*) (Van Bressem, 1994), bottlenose dolphins (*Tursiops truncates*) (Blanchard et al., 2001), a Blainville's beaked whale (*Mesoplodon densirostris*) (Saliki et al., 2006) and a dwarf sperm whale (*Kogia sima*) (Smolarek Benson et al., 2006).

1.3. Herpesviruses in pinnipeds

Bayesian phylogenetic trees of partial DNA polymerase sequences isolated from members of the families *Otariidae* and *Phocidae* cluster the seven pinniped herpesviruses within the subfamilies *Alphaherpesvirinae* (PhHV-1) and *Gammaherpesvirinae* (OtHV1,2; PhHV-2 - 5) (Maness et al., 2011).

Not much is known about most of the pinniped herpesviruses yet and published studies often are case descriptions or retrospective analyses of pathological findings. The isolates found to date vary regarding their symptomatology and characteristics of disease.

Otariid herpesviruses

OtHV-1 is a gammaherpesvirus that has been associated with the development of urogenital carcinoma in California sea lions (King et al., 2002). The incidence of neoplasia in those animals of the Pacific is high (Gulland et al., 1996) and OtHV-1 DNA could be detected in 100% of samples tested (King et al., 2002).

OtHV-2 was recently detected by PCR amplification and sequencing of an eye swab from a California sea lion and was shown to belong to the subfamily *Gammaherpesvirinae* (Maness et al., 2011).

Phocid herpesviruses

Phocid herpesvirus type 1 (PhHV-1), an alphaherpesvirus belonging to the genus *Varicellovirus*, is more closely described in the next section below.

PhHV-2 was described as a putative gammaherpesvirus isolated from European and North American harbor seals (Harder et al., 1996). Specimens from which virus was isolated were leukocytes and lung tissue (Harder et al., 1996; Lebich et al., 1994).

PhHV-3 and -4 were detected in nasal swabs from Hawaiian monk seals and classified as gammaherpesviruses (Goldstein, 2006a). 20% of both, seals in captivity and free ranging animals, were PCR positive with significantly higher numbers in animals in captivity – most likely indicating higher stress levels and consequent reactivation.

PhHV-5 sequences were found in mucosal swabs from a harbor seal and Bayesian phylogenetic analysis revealed a classification within the genus *Macavirus* in the gammaherpesvirus subfamily (Maness et al., 2011).

Table I-1: Phocid herpesviruses

Names of host species, herpesvirus, herpesvirus subfamily and GenBank accession numbers of glycoprotein B (gB) and glycoprotein D (gD) nucleic acid sequences of OtHV and PhHV

host species	herpesvirus	polymerase	subfamily	gB	gD
Zalophus californianus	OtHV-1	AF193617	*Gammaherpesvirinae*		
Zalophus californianus	OtHV-2	GQ429148	*Gammaherpesvirinae*		
Phoca vitulina	PhHV-1	U92269	*Alphaherpesvirinae*	U92270, S81228, Z68147	U92271, S81229, AJ290955
Phoca vitulina	PhHV-2	GQ429152	*Gammaherpesvirinae*		
Monachus schauinslandi	PhHV-3	DQ093191	*Gammaherpesvirinae*		
Mirounga angustirostris	PhHV-4	DQ183057	*Gammaherpesvirinae*		
Phoca vitulina	PhHV-5	GQ429153	*Gammaherpesvirinae*		

2.4. Phocid herpesvirus type 1 (PhHV-1)

Virus isolation, clinical disease and pathology

The first isolation and partial characterization of PhHV-1 was described from a disease outbreak in young harbor seals nursed in a seal orphanage in the Netherlands (Osterhaus et

al., 1985). Diseased animals were described to have symptoms of acute pneumonia and general depression, 11 out of 23 animals died. *Post-mortem*, acute pneumonia and focal hepatitis were seen in all animals with lethal disease. Seal kidney cells were used for virus isolation from organs and cytopathic effects could be detected *in vitro* and confirmed by immunofluorescence with serum from a seal that survived the outbreak. Viral particles were shown in negative contrast electron microscopy. Paired serum samples from ten surviving seals were tested in virus neutralization assays (VN) and showed seroconversion after 20 days. Crossreactivity with anti-canine herpesvirus (CaHV-1) and anti-feline herpesvirus (FeHV-1) serum but not with herpesviruses derived from other species (equine, bovine, suid, human) was evident in VN. From serological data and restricted cytopathogenicity it was concluded that the isolated herpesvirus was related to but distinct from CaHV-1 and FeHV-1.

A second report from the same outbreak from July / August 1984 emphasized clinical and pathological findings (Borst et al., 1986). Illness lasted for 1-6 days and symptoms were specific to the upper and lower respiratory tract (nasal discharge, inflammation of the oral mucosa and later coughing), to the digestive system (vomiting and diarrhea) and coming along with general depression including lethargy and anorexia. Main findings in necropsies were small ulcerations in the oral mucosa, lung emphysema and interstitial pneumonia with mononuclear infiltrations, dystrophic degeneration of liver parenchyma and degenerative changes in distal tubules of the kidneys.

During the big seal epizootic in the North Sea in 1988, phocine herpesvirus was first thought to be the potential causative agent (Frey et al., 1989). It was shown that PhHV-1 only played a minor role in diseased animals, however, and that herpesviral infections may have been secondary to immunosuppression in animals infected with morbilliviruses.

In 1994, the electron microscopic detection of herpesviruses in botryomycotic, granulomatous lesions of a captive harp seal with accumulation of virus in macrophages and CaHV-1 cross-reactive antibody titers was described (Daoust et al., 1994).

The first isolation of PhHV-1 from Pacific harbor seals was reported in 1997 (Gulland et al., 1997). Seven hundred live stranded harbor seal pups from January 1990 to December 1995 were included in a study and the clinical and post-mortem picture seen differed from what had been known from PhHV-1 cases in Atlantic waters. Multifocal acute to chronic adrenal necrosis was the most striking feature seen and was accompanied by hepatic necrosis and ulcerations of the oral mucosa in some cases. The high mortality led to the conclusion that PhHV-1 is an important cause of morbidity in seal pups in the Pacific, as well.

A retrospective study on PhHV-1 in wild and rehabilitating harbor seals in the northeastern Pacific (2000 - 2008) supported previous data (Himworth et al., 2010). All PhHV-1 positive seals were neonates or weanlings, adrenal necrosis was found in 95% of cases, hepatic necrosis in 41% and interstitial pneumonia only in 6%. Most animals (68%) were found with

pathology associated with other, unrelated disease processes such as bacterial pneumonia or enteritis.

First transmission studies were conducted with four to five month old harbor seals that were exposed with PhHV-1 intranasally and on the conjunctiva or alternatively exposed to animals infected one day later (Horvat et al., 1989). In two out of 15 seals, virus could be isolated from nasal swabs in animals with fever (directly inoculated animals) and low antibody titers were detected. The conclusion, that PhHV-1 is not transmitted horizontally was contradicted later on (Goldstein et al., 2004). Here, transmission was studied in free-ranging and rehabilitating Pacific harbor seals in California. Monitored exposure showed rapid horizontal transmission upon direct contact with nasal and oral secretions. Virus shedding in vaginal secretions and premature newborns with detectable early infections provided evidence for vertical transmission. PCR diagnostic in free ranging seals over a period of three years showed high prevalences only during the pupping time from May to August, supporting previous reports about age-restricted outbreaks in young animals. Prevalences in rehabilitated animals (54%) exceeded those in free-ranging animals (40%) not by much.

The first report on age-related differences in severity of disease and clinical signs described prevalences and clinical signs of PhHV-1 in harbor seals in rehabilitation during the years 1993 and 1994 (Harder et al., 1997). PhHV-1 was diagnosed serologically by virus neutralization of paired serum samples, by virus isolation on seal kidney cells and by PCR and Southern blotting with a glycoprotein D fragment probe. Adult animals were seropositive but no apparent infection could be detected. Juvenile animals of 1 to 12 months were seen with inapparent to severe forms of illness. In seal pups up to one month (neonates), disease was fatal in some cases and generally seen to be more severe when compared to older age groups. Benign upper respiratory disease was the most common clinical feature seen.

The inverse correlation of severity of clinical signs and age was specifically addressed in a long-term retrospective study (Martina et al., 2002). Clinical signs observed in rehabilitated harbor seals and grey seals from the years 1993, 1994 and 1998 were compared at different ages. Harbor seals generally developed more severe illness than grey seals. Consistent with earlier reports, severity of clinical signs was inversely correlated with age.

The tissue distribution of PhHV-1 in Pacific harbor seals was evaluated by PCR detection of a gB fragment (Goldstein et al., 2005). Stranded harbor seals of different age were examined histologically, serologically and by PCR of tissue samples. Herpesvirus DNA could be detected in various lymphoid and neuronal tissues, in some cases in the absence of apparent disease and generally more widespread than the presence of lesions. The detection of PhHV-1 in newborn animals with no contact to other infected seals again indicated that vertical *in utero* or perinatal infection may occur.

A decrease in PhHV-1 mortality occurred in the last years and is thought to be related to better husbandry protocols (less handling of animals leading to stress reduction) and improved veterinary care and quarantine (Goldstein et al., 2004).

Molecular characterization of PhHV-1

Monoclonal antibodies against two different PhHV-1 isolates were generated for taxonomic analyses and diagnostic purposes (Lebich et al., 1994). Antigenicity profiles of different harbor seal herpesvirus isolates in comparison to FeHV and CaHV data revealed a close antigenetic relationship of pinnipeds and terrestrial carnivores. One phocine herpesvirus isolate was less closely related and designated PhHV-2.

The panel of monoclonal antibodies was used again in a later study (Harder et al., 1996). Different virus isolates from European and United States coastal waters were compared antigenetically with each other and with feline and canine herpesvirus isolates. All but one European herpesvirus isolate clustered antigenetically with PhHV-1 whilst all but one US isolate were designated PhHV-2. For genetic analyses, partial gB and gD sequences could only be amplified from PhHV-1 and an *EcoR*I fragment was used for cloning and sequencing of PhHV-2 isolates. Comparison of similarity and identity values revealed that PhHV-1 clustered with members of the genus *Varicellovirus* (alphaherpesvirus), whereas PhHV-2 was more closely related to gammherpesviruses like Epstein-Barr-virus or equine herpesvirus type 2.

The molecular characterization of PhHV-1 glycoprotein B was conducted using a baculovirus - insect cell system for expression (Harder and Osterhaus, 1997). Studies revealed that the 881 aa protein is expressed as 113 kDa glycosylated molecule in mammalian cells, which is proteolytically cleaved into at least two fragments of 67 and 53 – 59 kDa, further forming disulfide-linked heterodimers of 140 kDa. After expression in insect cells, gB could be identified as 105 kDa glycosylated molecule and the proteolytic cleavage was delayed in recombinant baculovirus-expressed gB.

The antigenetic and genetic characterization of a herpesvirus isolated from a Pacific harbor seal identified it as PhHV-1 (King et al., 1998). Sequence fragments of the DNA dependent DNA-polymerase, gB and gD were compared. More small nucleotide polymorphisms (SNPs) were seen here than when European PhHV-1 isolates were compared, resembling the co-evolution of virus and harbor seal sub-species (Stanley HF 1996).

The complete unique short segment of PhHV-1 was cloned and sequenced and shown to comprise glycoproteins gG, gD, gI and gE, a putative protein kinase and the homologous ORF of HSV's Us2, Us8.5 and Us9 (Martina et al., 2003). Phylogenetic analyses revealed a clustering within the subfamily *Alphaherpesvirinae* and closest relationship to canids and, to a lower extent, felids.

As described earlier, phylogenetic analyses of all to date discovered marine mammal herpesviruses by the comparison of DNA-polymerase sequences were published recently (Maness et al., 2011).

Therapeutic approaches

No treatment was available for outbreaks of PhHV-1 in the Dutch seal orphanage, where PhHV-1 was first isolated and a recurrent problem. Different antiviral compounds were therefore tested, some of which were known for their efficacy, others just broad-spectrum antiviral drugs. Two compounds were found to be effectively inhibiting the cytopathogenic effects *in vitro* (Osterhaus et al., 1987), no further *in vitro* studies were published later and morbidity and mortality rates diminished with better veterinary care and quarantine protocols.
Major immunogenic glycoproteins of phocine herpesviruses were investigated in radioimmunoprecipitation assays and gB was not only found to be the most immunogenic but also the antigenically highest conserved protein when compared to CaHV and FeHV. Envelope glycoproteins of herpesviruses are prominent targets of cellular and humoral immune responses (Jennings et al., 1998) and it was concluded that gB was a sensible component of future PhHV-1 subunit vaccines (Harder et al., 1998). The development of an anti-PhHV-1 vaccine was perpetuated and a PhHV-1 based ISCOM vaccine was compared with FeHV and CDV (canine distemper virus) based ISCOM vaccines for protection of cats against FeHV *in vivo* (Martina et al., 2001). After challenge, all cats were protected from severe disease and virus shedding was reduced significantly.
PhHV-1 gB and gD were expressed in insect cells using a baculovirus expression system and combined gB/gD containing ISCOM vaccine candidates were compared to gB and gD only ISCOM and a mock control in BALB/C mice (Martina et al., 2006). Challenges revealed higher cellular and humoral immune responses and levels of protection in mice immunized with vaccine candidates directed against both glycoproteins.

Serological surveys of free ranging pinnipeds in the Northern hemisphere

Seroprevalences for phocid herpesvirus were first investigated for harp and hooded seals in the West Ice north of Jan Mayen and in the Barents Sea, East Ice in the spring of 1991 and 1992 (Stuen et al., 1994). Blood samples from harp seals were collected in both areas and revealed 28.6 - 41.1% positive animals. Hooded seals were sampled only in the West Ice and seropositivity was lower with 4.3 - 6.7%. This data may reflect the occurrence of aerosol transmission related to the social behavior of the different species: Harp seals stay in large groups in water and on land during parturition and molting compared to a more solitary life style of hooded seals, forming small groups of two to three animals during pupping and molting season (King, 1983).

The prevalences of PhHV-1 and PhHV-2 in different pinniped species were investigated in a serological survey off the coast of Alaska and Russia during 1978 – 1994 (Zarnke et al., 1997). Harbor seals sampled in Southeast Alaska, Prince William Sound, Cook Inlet, Kodiak Island, the Alaska Peninsula and Probilof Islands (all locations along the southern coast of Alaska and in the Bering Sea) were shown to be seropositive for PhHV-1 with 77% and for PhHV-2 with 42%. Seroprevalences for other species (walrus (*Odobenus rosmarus*), Northern fur seal (*Callorhinus ursinus*), spotted seal (*Phoca largha*), ribbon seal (*Histriophoca fasciata*), Steller sea lion (*Eumetopias jubatus*), bearded seal (*Erignathus barbatus*), ringed seal (*Pusa hispida*)) ranged between 29% and 72% seropositivity for PhHV-1 and between 17% and 50% for PhHV-2 respectively. Lower prevalences of PhHV-2 were assumed to be linked with the high degree of cell-association of the virus and consequently a different route of transmission than aerosol as in PhHV-1.

An indirect enzyme linked immunosorbent assay (ELISA) for PhHV-1 specific antibodies was developed to evaluate the serological status of Pacific harbor seals (*Phoca vitulina richardsii*) stranding at the coast of California in February to August of the El Nino event year 1998 (King et al., 2001). Large numbers of stranded animals with high antibody titers occurred in April to May with up to 89% seropositive animals. Later in summer, fewer animals were admitted to the rehabilitation center but all were seropositive for PhHV-1. Interestingly, 82.4% of pups dying with symptoms of PhHV-1 were seronegative before death, indicating that the disease progresses faster than a humoral immune response can be developed.

A broader survey evaluated whether PhHV-1 was endemic in harbor seals from both coasts of North America from 1994 to 2002 (Goldstein et al., 2003). In free ranging seals, prevalences were comparable between coastal waters off Alaska, the Northwest, the Southwest and the Northeast (90 – 98%). Significantly fewer animals had anti-PhHV-1 antibodies when held in rehabilitation (23 – 38%) and all animals in permanent facilities were seropositive. No differences were seen between gender, but prevalences increased with age from 37.5% in pre-weaned pups, 87.6% in weaned pups to 99% in subadult and adult harbor seals.

Serological surveys of free ranging pinnipeds in the Southern hemisphere

Only two reports of PhHV-1 prevalence in Antarctic pinnipeds are published to date. Interestingly, samples analyzed in both of the studies were derived from the same expedition (German Weddell Sea expedition ANT VIII of the research vessel “Polarstern” in January/February 1990) but revealed contrary results.

In the first report, sera of 25 Weddell seals and 3 crabeater seals were tested (Harder, 1991). Weddell seals were seen to suffer from respiratory disease at the time blood samples were taken. All animals were shown to be seropositive in microneutralization assays.

One year later, neutralization assays on samples (18 Weddell seals and 2 crabeater seals tested) were reported to show no detectable antibody titers in crabeater seals and 72% seropositivity in Weddell seals (Stenvers et al., 1992).

2. The former order "pinnipedia"

The term "pinniped" is derived form the Latin *pinnipes* = wing-/fin-footed, referring to the characteristic extremities of animals formerly classified within this order (Reeves, 2009). Today, the three closely related families of *Ontariidae* (eared seals), *Phocidae* ("true" seals) and *Odobenidae* (with one single species, the walrus) are classified within the order *Carnivora*, the term pinniped is still frequently used for the description of those closely related, carnivore mammals inhabiting the marine environment.
The pinniped's main body characteristics are a large body with paddle- or fin-like modified appendages, a short snout and large eye sockets and eyes and a thick subcutaneous fat layer, the so-called blubber layer. Those features adapt them to their aquatic lifestyle, allowing them to swim efficiently and still move on land. A good insulation is important since temperature conduction is about 25 times higher in water than in air. Animals molt once a year, usually in summer or fall. Breeding social structures range species-specific from polygyny to monogamy. Some species form large colonies during the breeding season, while others, mostly ice-breeding phocids, stay more solitary during that period. Pinnipeds breed shortly after giving birth to usually one single pup. To allow parturition, lactation and mating within such a short time frame, pinniped embryos undergo embryonic diapause, which is a delayed implantation due to the energy demands on the female body.

Closer descriptions of different species are only focused on pinniped species sampled for studies described later and do not claim to be a complete summary of all pinniped species of the world.

2.1. The family *Ontaridae*

Sea lions and fur seals are classified within the family *Ontaridae*, also known as eared seals (Reeves, 2009). This name derives from the fact that they have, unlike members of the family *Phocidae*, outer ear pinnae. At least 15 species within 7 genera are known.
Besides their characteristic earflaps, ontarids can be distinguished from phocids by the build of their extremeties. Ontarid's fin-like extremities enable them to walk on land with both their

for- and hindflippers, being able to rotate their hindlimbs underneath their body. Propulsion when swimming is mainly derived from their forelimbs. Their locomotion on land reminds more of terrestrial carnivores and their underwater movements are more similar to those seen in penguins than in fish.

Fur seals, unlike sea lions, have a layer of short and dense underfur underneath a layer of longer outer hair, trapping air and contributing to insulation.

Seven members of the family *Ontaridae* live in the Southern hemisphere, all other fur seal species are located in the northern Pacific.

2.2. The family *Phocidae*

True or earless seals lack external ear flaps and include 18 species and one extinct species in 13 genera (Reeves, 2009).

Their body is, compared to ontarids, more adapted to aquatic life and their hind flippers cannot be used for locomotion on land. They are mainly gliding on ice by pulling their body with their front extremities whilst in water using them only for steering. Propulsion in water comes from moving their strong, widespread hindlimbs from side to side.

Phocids have a particularly thick layer of blubber, not only for insulation but also serving as energy reserve during periods of fasting.

Phocids are distributed over both hemispheres with five species living circumpolar around the Antarctic continent and the others distributed in the North Atlantic, the Arctic or living highly localized.

Ontarid and phocid species, which were part of the study, are listed in table I-2.

Table I-2: Seal species and habitat

Occurrence and characteristic behavior/ distribution of seal species found in our study

***Ontaridae*: species**	**habitat**	**characteristics**
Antarctic fur seal (*Arctocephalus gazella*)	Antarctica during winter months almost exclusively at sea	males living in groups during molt, females strongly solitary

Phocidae: species	habitat	characteristics
Harbor seal (*Phoca vitulina*)	Arctic and sub-arctic regions in the North-Atlantic and Pacific	mostly living solitary with the exception of gathering in big groups for molting on land
Harp seal (*Pagophilus groenlandicus*)	pack ice of the North Atlantic between Newfoundland and Russia	migrating and feeding in large groups even after molting and breeding
Hooded seal (*Crystophora cristata*)	central and western North Atlantic along the edge of the pack ice zone	living solitary, shortest mammalian mother-pup period (4 days)
Weddell seal (*Leptonychotes weddellii*)	fast ice zone (attached to the shore) around Antarctica	living mostly solitary, males competitively defending territories under the ice
Ross seal (*Ommatophoca rossii*)	pack ice around Antarctica, mostly Ross Sea and King Haakon VII Sea	living solitary; not much is known due to inaccessible habitat
Crabeater seal (*Lobodon carcinophagus*)	pack ice around Antarctica	found solitary or in smaller or larger groups
Southern elephant seal (*Morounga leonina*)	subantarctic oceanic islands and Southern Oceans	molting and mating in large groups, foraging solitarily

2.3. Ocular disease in pinnipeds

Ocular lesions are a common finding in both free-ranging and captive pinnipeds (Dierauf, 2001; Schoon and Schoon, 1992). Particularly, animals in captivity are frequently observed to show clinical signs of conjunctivitis, keratitis, corneal edema, ulceration and cataracts (Hirst, 1983). The incidence in wild animals appears lower than in captive seals (Stoskopf, 1985). Only little evidence on cases and incidence of ocular disease in pinnipeds can be found in the scientific literature, however, and etiology and pathogenesis remain obscure (Dierauf, 2001; Griner, 1983).

A total number of 126 captive seals from different European zoos and marine lands were examined for signs of anterior segment ocular disease. Animals were of 7 different species and 22.2% showed clinical signs of ocular disease (Greenwood, 1985).

A study, in which 1,716 wild Northern fur seals (*Callorhinus ursinus*) were examined for ocular symptoms revealed clinically recognizable eye disease in 4.6% of animals with corneal scars being the most frequent finding (Stoskopf, 1985). During the morbillivirus epidemic (phocine distemper virus, PDV) during 1988/89 in the North Sea, lenticular lesions were detected in 21 of 44 harbor seals. Exogenous causes such as trauma were excluded due to the bilateral occurrence and there was no convincing evidence for the involvement of

infectious agents such as PDV (Schoon and Schoon, 1992). In an investigation of the highly endangered species Hawaiian Monk Seals (*Monachus schauinslandi*), the progression of ocular disease in a group of seals captured for research purposes was reported. Eleven out of 12 seal pups developed signs of disease over time. First clinical signs were ocular discharge, red eyes, conjunctivitis, photophobia and blepharism; affected animals later developed corneal edema, bullous keratitis and severe corneal opacities. Whilst opacities were seen unilaterally first, all affected animals showed bilaterally opaque eyes later on (Hanson, 2009).

An increased incidence of ocular disease in captive seals has been discussed in the context of freshwater environments (Dunn, 1996) and other risk factors such as by-products of chlorine disinfection, high incident UV light, pH imbalances and opportunistic pathogens (Dierauf, 2001). This is going along with the report of increased incidences during the PDV epidemic (Schoon and Schoon, 1992), pointing to a multifactorial etiology of ocular disease in pinnipeds.

3. Aims of the study and working hypothesis

The following study was conducted to investigate the occurrence of PhHV-1 in seals in different environments. Herpesviruses are one of the major pathogens affecting pinniped health in the wild and in captivity. Testing of animals, particularly in the wild, is difficult and only few studies on PhHV-1 in free ranging seals have been conducted to date. A large number of seal species are inhabiting mainly the Polar regions and therewith some of the most hostile environments worldwide, some of which have never even been tested for PhHV-1 prevalence. With new diagnostic methods and the acquirement of more data we hope to contribute to a better understanding of the disease and its impact on the marine ecosystem.

Two main questions were addressed in the context of PhHV-1 infections:

Firstly, a qPCR assay was established for the detection of PhHV-1 DNA, facilitating sensitive and specific detection of viral nucleic acid in swab samples. The main focus was the detection of PhHV-1 in ocular swabs. Lenticular lesions are frequently seen in pinnipeds and are of major concern for zoos, marine parks and rehabilitation facilities. The etiology of the disease is still unknown and treatment is only symptomatic. This study addresses the question, whether herpesviruses may play a role in the etiology of anterior segment ocular disease in pinnipeds. The investigation of wild animals allows the exclusion of factors associated with the captive environment such as chemical and physical parameters of the

water or increased solar UV radiation. The assay could further be used for the detection of PhHV-1 DNA in nasal and genital swab samples.

Secondly, to complete the picture and to add data to the current knowledge about prevalence of the disease in different species and environments, a PhHV-1 ELISA was developed and serological surveys were conducted in different populations inhabiting the Northern and Southern hemisphere.

Chapter II – MATERIALS AND METHODS

In the second chapter, materials and methods used for the study are listed and described. Information regarding sampling of animals and maintenance of samples was kindly provided by Drs. Morten Tryland (Section of Arctic Veterinary Medicine, Norwegian School of Veterinary Science, Tromsø, Norway), Ingebjørg H. Nymo (Section of Arctic Veterinary Medicine, Norwegian School of Veterinary Science, Tromsø, Norway) and Susana P. Díaz (Department of Animal Health, Faculty of Veterinary Medicine, Madrid, Spain).

Some of the information and data found in this study is intended to be published in the following manuscripts (also indicated at the respective paragraphs in the text):

Serum chemistry and antibodies against pathogens in Antarctic fur seals, Weddell seals, crabeater seals and Ross seals
Morten Tryland, Ingebjørg H. Nymo, Ole Nielsen, Erling S. Nordøy, Kit M. Kovacs, Bjørn A. Krafft, Stein I. Thoresen, Kjetil Åsbakk, Nikolaus Osterrieder, Swaantje J. Roth, Christian Lydersen, Jacques Godfroid, Arnoldus S. Blix
Submitted to J. Wildl. Dis., June 8th 2011, accepted

Detection of phocid herpesvirus 1 DNA in swab samples of free ranging harbor seals from Svalbard by qPCR and long-term serosurveillance
Swaantje J. Roth; Nikolaus Osterrieder; B. Karsten Tischer; Kit M. Kovacs, Christian Lydersen, Morten Tryland
In preparation

Detection of antibodies against phocid herpesvirus type 1 (and....) in pinniped species from the Antarctic Peninsula
(…), Swaantje J. Roth, Nikolaus Osterrieder, Susana P. Díaz
In preparation

A detailed list of materials, equipment, reagents and solutions including the composition of solutions and manufacturers can be found in the appendix (pp.xviii-xxiv) and will not be given in the text.

1. Sampling of animals and maintenance of samples

All fieldwork was conducted by experts from

- the Section of Arctic Veterinary Medicine, Norwegian School of Veterinary Science, Tromsø (Norway)
- the Norwegian Polar Institute, Tromsø (Norway)
- the Department of Arctic and Marine Biology, BFE, University of Tromsø (Norway)
- the Department of Animal Health, Faculty of Veterinary Medicine, Madrid (Spain).

1.1. Arctic specimens

Norwegian expeditions to Svalbard 1998, 1999, 2000, 2009, 2010

(harbor seals)

Blood samples of harbor seals were obtained in 1998 (n = 59), 1999 (n = 74), 2000 (n = 81), 2009 (n = 87), and 2010 (n = 83) from the world's northernmost colony of this species, at Prins Karls Forland (78° 15′ N, 12° 10′ E), on the west side of Spitsbergen, Svalbard (Figure II-1) (Roth, manuscript in preparation).

Figure II-1: Map of the Svalbard archipelago

Blood and ocular and nasal swab samples were obtained from free-ranging and presumably healthy harbor seals (*Phoca vitulina*) on Prins Karls Forland (78° 15′ N, 12° 10′ E), on the west side of Spitsbergen, Svalbard; figure 1 from (Roth, manuscript in preparation)

Nets for capturing were set around haul-out sites. Body weights of animals were detected with a spring scale (Salter Brecknell, MN, USA) and seals were sedated with zolazepam and the dissociative/tiletamine (Zoletil®: 0.5–1.0 mg/kg body mass). Extraction of an incisor tooth from each animal was conducted for age determination, using the method of counting cementum layers in decalcified and stained longitudinal sections of the teeth (Lydersen, 1987). Blood was drawn from the extradural intravertebral vein and samples were centrifuged at 1 800 x g for 15 min, and serum was stored at – 20°C until analysis. Sterile cotton swabs (SelfaTrade, Spånga, Sweden) and sterile Dacron swabs (NerbePlus, Winsen/Luhe, Germany) were used for sampling (2009; n = 87, 2010; n = 83) from one eye (inside lower eye lid) and 3-4 cm inside one nostril from each animal during sedation. Swabs were kept in sterile cryotubes with 800 µl cell culture medium (Eagles Minimal Essential Medium; EMEM) containing antibiotics (penicillin (100 U/ml) and streptomycin (100 µg/ml), gentamicin 50 µg/ml and amphotericin B 2.5 µg/ml) at – 20°C in the field, and transferred to – 80 °C after a maximum of 3 weeks after sampling until analysis.

Permission to chemically immobilize and sample harbor seals was given by the Governor of Svalbard (Sysselmannen) and animal-handling protocols were approved by the Norwegian Animal Research Authority.

Norwegian expeditions to the West Ice 2009 and 2010
(hooded and harp seals)

Hooded seals from the northeastern Atlantic population were lethally captured for research purposes only on a commercial hunting vessel in April / May 2009 in the West Ice between Greenland and Jan Mayen island, North of Island. All animals were below one month of age, since they were found solitary on the ice which they are known to do for only four weeks after weaning (Kovacs, 1990). Animals were euthanized according to Norwegian law and regulations and weight, length and gender were determined. Organ and tissue samples were taken and kept frozen at −20°C until further use. Genital (n = 71), ocular (n = 71) and nasal swabs (n = 72) were taken using dry, sterile cotton tip applicators (Selefa trade, Sweden). Sampling was done by rubbing the cotton tip applicator gently against the mucus membranes. Sampling was not done if large amount of blood were present. Swabs were stirred vigorously in 1.8 ml cryotubes with MEM and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B) and then removed. Samples were stored at −20°C and later −80°C until analysis.

Harp seals were lethally captured during the same expedition for commercial hunting reasons and genital, ocular and nasal swab samples, n = 174, n = 175 and n = 175 respectively, could be obtained. Again, animals with large amounts of blood were excluded from the sampling procedure, no age data is available for harp seal samples.

In 2010, during a research expedition in the same region, n = 20 harp seals, n = 151 hooded seals and one bearded seal were lethally captured. Gender, body length and body weight were determined in all animals and ocular and nasal swabs and blood samples were taken from hooded (n = 20) and harp seals (n = 9). From hooded seals, 20 ocular swabs and 17 nasal swabs were obtained, both ocular and nasal swabs could be taken from all nine harp seals. Scientific (investigation of the reproductive status of the harbor seal population) and hunting quotas were given to the Institute for Marine Research by the Norwegian Government and animal-handling protocols were approved by the Norwegian Animal Research Authority.

1.2. Antarctic specimens

Norwegian expedition 2000/2001

(Antarctic fur seals, Weddell seals, Ross seals, crabeater seals)

In the months December 2000 and January 2001 (n = 52) and January 2002 (n = 35), Antarctic Fur Seals were sampled on the shore of Bouvetøya (Norway; 54.41°S, 03.29°E) (Tryland, submitted June 8th 2011). Animals were immobilized chemically with tiletamine-zolazepam (100 mg/ml; Zoletil®, Reading, L'Hay-Les-Roses, France). Bulls (n = 10) were injected intramuscularly with a 10 ml syringe attached to a rod, adult females (n = 46) were captured and immobilized on a custom built restraining board. Weights of adults were determined with a Salter spring scale, bulls in an A-frame net and females on the restraining board. Pups (n = 31) were categorized based on an evaluation of size, naïve pup behavior and absence or status of the umbilical cord. They were weighed in a canvas bag. Standard body length was measured for all animals according to the Committee on Marine Mammals (1967) and all were tagged in the front flippers using plastic ear tags for cattle (Dalton Jumbo Tag, Dalton Supplies, Henley-on-Thames, Oxon, UK). Blood was drawn from the extradural intravertebral vein with a 30 ml syringe and transferred into 10 ml vacutainer blood tubes. Serum was separated by centrifugation within 3 h of sampling at 1500 x g for 10 min and then kept frozen at −20°C until analysis. All seals appeared to be in good general health. However, all bulls had bite wounds, scars or fresh or infected wounds on the front flippers and in the head, neck and thorax regions.

Blood was collected from 20 adult Weddell Seals (10 males, 10 females), 20 adult Ross Seals (11 males, 9 females) and 10 adult crabeater seals (2 males and 8 females) from King Haakon VII Sea, off Queen Maud Land, Antarctica, in January and February 2001. The animals were immobilized by intramuscular injection of Zoletil® (Weddell seals 2.5 – 3.0 ml, Ross seals 1.0 – 2.0 ml, crabeater seals 2.5 ml) delivered by use of a dart gun. Blood was

collected in syringes by way of a 45 mm polyethylene catheter (16G Venfon, Viggo AB Helsingborg, Sweden) inserted into the extradural intravertebral vein. Serum was prepared and frozen at −20°C until analysis. All animals appeared to be in good health.

The capture of animals was done under permit from the Norwegian Polar Institute and sampling was carried out under permit from the Norwegian Animal Research Authority (NARA).

Spanish expedition 2010

(Weddell seals, Southern elephant seals, Antarctic fur seals)

A total of 80 animals from different pinniped populations from Deception Island and other areas in the South Shetland Islands and Antarctic peninsula were sampled during the month of February 2010 (Díaz). Included were samples from Weddell seals, Southern elephant seals, and Antarctic fur seals. Animals were captured and physically restrained according to standard procedures. Blood samples were collected, centrifuged and the sera were stored at −20° C until analysed. In addition, 70 nasal swabs were collected from the same animals by insertion of sterile cotton swabs into the nose. Swabs were kept in sterile PBS with antibiotics at −20° C until arrival at the laboratory and stored at −80° C from then on. No information on age, body measurements or gender was collected.

DNA from nasal swabs was extracted from 200 µl sample aliquots using a BioSprint96 workstation (Qiagen) and the BiosSprint DNA blood kit (Qiagen) according to the manufacturer´s instructions.

2. Cells and viruses

2.1. Cells

Crandell Reese Feline Kidney cells (CRFK) were propagated in Eagle's minimal essential medium (MEM) supplemented with 5 - 10% fetal bovine serum (FBS). In order to keep cells growing in log phase, they were regularly split 1:2 – 1:8. To split cells, media was removed and cells were detached from the cell culture flask or dish by incubation (37°C) in 0.04 ml/cm^2 trypsin-EDTA for about 3 min. Loose cells were carefully resuspended in MEM 10% FBS and reseeded at desired densities.

For long-term storage, cells were frozen in aliquots of 10^6 – 10^7 cells. Cells were resuspended in approximately 1 ml of MEM 10% FBS after trypsinizing and then mixed with MEM/FBS/DMSO to a final concentration of 10% dimethyl sulfoxide (DMSO) and 20 – 30%

FBS. As soon as cells were exposed to DMSO, they were aliquoted quickly and frozen slowly to a final temperature of −80°C with the help of a cryo storage system. Once they reached the final temperature, aliquots were transferred into liquid nitrogen for long-term storage.
To recover cells from liquid nitrogen long-term storage, aliquots were quickly thawed in a 37°C waterbath, each aliquot was resuspended in 10 ml of fresh MEM 10% FBS and pelleted though centrifugation at 300 x g for 5 min at RT. Cells were then diluted in the appropriate amount of medium and seeded in 75 cm^2 cell culture flasks.

2.2. Virus

A PhHV-1 isolate (most likely isolate 2577/Han88, isolated from leukocytes of diseased harbor seal in 1988, a kind gift of L. Haas, Tiermedizinische Hochschule, Hannover, Germany) was grown on CRFK cells. For the production of virus stocks, cells were infected at a multiplicity of infection (MOI) of about 0.1. Infected cells show characteristic cytopathogenic effects (CPE, plaques) and were harvested after 3 days. For harvesting of virus containing supernatants, cells were frozen (-80°C) and thawed (37°C) three times, cell debris was removed by centrifugation (1500 x g for 15 min at RT) and clear supernatant containing virions was frozen in 0.5 – 1 ml aliquots at −80°C.
Titers were determined by plaque assays. 12- or 24-well plates with CRFK cell monolayer at a density of approximately 90% were infected with a defined volume of serial 10-fold dilutions of virus stocks (10^0 – 10^{-5}) in duplicates. Carboxymethylcellulose in MEM (0.5 – 1 ml of 1.6%) was added to each well 60 – 120 min post infection. At 48 h after infection, cells were washed with PBS, dried and fixed with ice-cold methanol:acetone (v/v) 1:1 at -20°C for 15 min. Monolayers were dried again after fixation, stained with crystal violet solution for 10 min at RT and washed with water several times before final drying. Plaques were counted macroscopically in wells with 10 – 100 plaques and the final number of plaque forming units (PFU) per ml was calculated considering the dilution and the volume of initial inoculation.
For isolation of virions, 1 x 10^8 CRFK cells were infected at an MOI of approximately 0.1. Forty eight h later, supernatant of infected cells was harvested and cell debris was removed by centrifugation for 10 min at 180 x g first and in a second step for 10 min at 1600 x g. Virions were then pelleted by ultracentrifugation for 60 min at 12°C and 125000 x g and the pellet of virions was resolved in a total volume of 1ml of ELISA coating buffer (0.013M Na_2CO_3; 0.037 M $NaHCO_3$; pH 9.5).

3. Molecular methods

3.1. PCR amplification of PhHV-1 sequence fragments

Primers for the amplification of PhHV-1 sequence fragments were designed with the help of VectorNTI software (Invitrogen), based on available sequence data (GenBank, NCBI). Primers for a glycoprotein B (gB) fragment were based on the sequence GenBank accession no. S81228 and primers for a fragment of the DNA dependent polymerase (Pol) were based on the sequence GenBank accession no. U92269. Primer sequences are given in table II-1.

Table II-1: List of primers and probe used in this study

primer / probe ID	sequence	description
SJR#03	GATATTGGAGATGTTCCAGAATC	Pol_fw
SJR#04	ACCATTGGAAACTCCTGTAAA	Pol_rv
SJR#09	ATCGTACGGGAACATCTG	gB_fw
SJR#10	CCCAACTTGTAGTCTGGTATCC	gB_rv
SJR#17	GGTTCTCGTGGATGGCATAC	gB_qPCRfw
SJR#18	CAGATCTGGCATCAACTTCT	gB_qPCRrv
SJR#b	ATCGTACGGGAACATCTG	gB_probe FAM/tamra

PCRs were conducted using supernatant of virus infected cells or viral DNA (II-3.4.) as template. Supernatant was incubated with Proteinase K (500 µg/ml) at 65°C for 20 min and the enzyme was then heat-inactivated at 95°C for 10 min. RNaseA was added to the supernatant at a final concentration of 100 µg/ml and supernatant was incubated at 37° C for 30 min. Primers were used as 10 µM working stocks to a final concentration of 0.2 µM each and dNTPs were used as 10 mM each solution to a final concentration of 0.2 mM. Longamp high fidelity Taq DNA polymerase and corresponding buffer were used according to the manufacturer's instructions.

PCRs were run under following conditions:

Initial denaturation		94°C	4 min
25 cycles of	denaturation:	94°C	30 sec
	annealing:	52°C	30 sec
	elongation:	65°C	50 sec / kb
final extension		65°C	7 min
hold		4°C	

Successful amplification of desired fragments was verified by agarose gel elecrophoresis, using 1% agarose mini gels in 0.5X TAE, containing 0.005 % ethidium bromide, run at 100 V for 30 min. Separated DNA was visualized under UV light.

3.2. Cloning of PhHV-1 sequence fragments

PCR fragments with TA overhangs were TA cloned into the vector pCRII using the Topo TA cloning kit (Invitrogen) following the manufacturer's instructions. In detail, 0.5 µl vector, 0.5 µl salt solution and 2 µl PCR product were mixed and incubated at RT for 5 min. The cloning reaction was further transformed into chemically competent *E. coli* TOP10 (preparation of chemically competent bacteria is described in II-3.3.). For chemical transformation via heat shock, the cloning reaction was added to one vial chemically competent TOP10 (thawed at RT and immediately kept on ice after), chilled on ice for 20 - 30 min, heat shock was performed at 42°C in a thermal incubator for 30 sec and tubes were immediately transferred back on ice. 750 µl SOB (super optimal broth) was added and recovery was carried out shaking at 37°C for 1 h. After recovery, 100 µl and the remaining volume of bacteria suspension (after concentration via centrifugation for 3 min at 845 x g and resuspension in 100 µl) were each spread on lysogeny broth (LB) - agar plates containing selective plates containing ampicillin (0.1 mg/ml) and incubated at 37°C over night.
For screening, single colonies were picked, grown and DNA was isolated as described in II.3.4., restriction digests were carried out as described in II.3.5. and DNA from several clones was sequenced after purification as described in II.3.6..
Plasmids were named pCRII.gB (SJR#7 and SJR#8) and pCRII.pol (SJR#3 and SJR#10). SJR#8 was further used as control plasmid for qPCR.

3.3. Preparation of chemically competent bacteria for transformation

250 ml SOB were inoculated 1:100 with a fresh culture of *E. coli* Top10 and incubated in a shaking incubator (220 rpm) at 18°C to an OD_{600} of 0.6. The culture was transferred into 50 ml falcon tubes and chilled on ice for 10 min. After centrifugation at 2500 x g for 20 min (4°C), bacteria pellets were carefully resuspended in 80 ml ice cold Tris-borate buffer (TB) and chilled for 20 min on ice. After a second centrifugation at 2500 x g for 20 min (4°C), pellets were resuspended in 20 ml ice cold TB, DMSO was added to a final concentration of 7% and aliquots of approximately 200 µl were immediately stored at −80°C until further use.

3.4. DNA extraction of plasmid and viral DNA

Plasmid DNA was extracted using standard alkaline lysis protocols (Sambrook, 2001). Briefly, 2.5 – 3 ml bacterial culture, grown at 37°C in a shaking incubator for 8 - 16 h, was pelleted for 5 min at 20200 x g and kept on ice for following steps. Pellets were dissolved in 100 µl Solution 1 (buffer P1). Two hundred µl Solution 2 (P2) were added and samples were inverted 10 times and kept lysing on ice for 3 min. Lysis was stopped by the addition of 150 µl Solution P3. Tubes were inverted 10 times again, kept on ice for a minimum of 5 min and after 5 min of centrifugation at 9400 x g, supernatants were transferred and mixed with 500 µl phenol / chloroform by inverting 10 times. Samples were centrifuged for 5 min at 20200 x g, supernatants were carefully transferred to new tubes and plasmid DNA was precipitated by the addition of 450 µl isopropanol, inversion and centrifugation at 20200 x g for 5 – 10 min. Pellets were washed with 200 µl ice-cold ethanol (EtOH) 70%, dried and dissolved in 30 µl TE (Tris-EDTA)- RNaseA (40 µg/ml) and incubated at 37°C for 30 min.
Bacterial plasmid DNA for qPCR standard curves was isolated using „Invisorb Spin Plasmid Mini Two“ (Inivitek, Berlin, Germany) according to the manufacturer’s instructions.
For viral DNA extraction, a T75 cell culture flask of CRFK cells was infected with PhHV-1 at an MOI of approximately 0.1 and harvested after 36 h by freezing (–70°C) and thawing (37°C) for three times. Cell debris was pelleted for 15 min at 2880 x g at RT and supernatant was further incubated with 1 % SDS for 15 min at 65° C. After RNaseA treatment (50 µg/ml) for 30 min at 37° C, proteinase K was added (500 µg/ml) and incubated at 37°C over night. The following day, supernatant was gently mixed with 2 ml phenol/chloroform (inverting tubes 20 times), kept at 4°C for 10 min and separated in centrifugation at 4500 x g and 4°C for 5 min. The upper phase was carefully transferred to a new tube and the step was repeated. Finally, the upper phase was transferred again and DNA was precipitated with 1/10X volume NaCl and 1X volume isopropanol at 4°C for 30 min (4500 x g). The DNA pellet was washed with 70% ice cold EtOH once and finally dissolved in 50 µl TE.

3.5. Restriction digest

Restriction digest were carried out in order to verify cloning success. Generally, 2 µl of plasmid DNA was added to 2 µl of respective buffer (NEB) and 5 units of restriction enzyme in a total volume of 20 µl and incubated at 37° C for 1 h. DNA fragments were run on 1% agarose gels and visualized as described for PCR products in II.3.1..

3.6. Sequencing of DNA

Plasmids were sequenced using the service of StarSEQ U-Mix (www.starseq.de, Mainz; ABI 3730 capillary sequencer). For sequencing analysis of plasmids, 3 µl miniprep DNA were mixed with 1 µl primer (10 µM) in a total volume of 7 µl. DNA of pCRII topo clones containing the respective PhHV-1 fragments was sent off for sequencing with primers T7 (KTL#15 TAATACGACTCACTATAGGG) and M13rev (KTL#13 CAGGAAACAGCTATGAC).
Obtained sequences were analysed and compared to reference sequences using the software VectorNTI ContigExpress (Invitrogen) and VectorNTI AlignX (Invitrogen).

3.7. Cryoconservation of bacteria

After restriction analysis and sequencing of DNA, respective bacterial clones were frozen for long-term storage at -80° C. 500 µl of fresh bacterial culture grown in LB with selective antibiotic (ampicillin 0.1 mg/ml) was mixed 1:1 v/v with sterile 50% glycerol in cryo-conservation tubes and glycerol stocks were stored at -80° C.

3.8. Quantitative real-time PCR (qPCR)

The genome regions of gB, gD and DNA polymerase (Pol) from published sequences (NCBI GenBank) and from the control plasmid were aligned using VectorNTI (Invitrogen). As later described (under III.3.), gB appears, at least regarding the available sequence data, to be more conserved than Pol. Additionally, more sequences from different isolates are available for gB, allowing a better prediction of conserved parts of the sequence used for primer and probe design. gB-specific primers and probe for qPCR were consequently designed with the help of the software primerExpress.3 (ABI) and VectorNTI (Invitrogen). All target sequences were chosen to be located within a highly conserved region of gB whilst fulfilling requirements for qPCR primer and probe design. Shortly summarized, the length of the amplicon of qPCR was aimed to be 50 - 150 bp (in this case 115 bp). Primer melting temperatures (T_m) should ideally be at or very close to 59°C (and both primers should be within the range of 1°C). The melting temperature for the probe is desired to be about 10°C above the primer melting temperature and target sequences for the probe should be located in close proximity to the primers, particularly the distance between 3' end of the probe and 5' end of the reverse primer should be kept very short.
An alignment of PhHV-1 gB sequences from the isolate compared with available Atlantic and Pacific isolates (NCBI GenBank) is shown in figure II-2. Binding regions for primers (SJR#17

and SJR#18) and probe (SJR#b) are indicated, binding to highly conserved nucleotide regions.

Figure II-2: Alignment of gB fragments with primer and probe binding sites

Primer and probe binding sites are black, underlined and marked in bolt

Firstly, optimal conditions for the PCR were determined. Therefore, primer concentrations were initially tested at five different dilutions each in a conventional PCR assay.

Table II-2: Primer dilutions for PCR

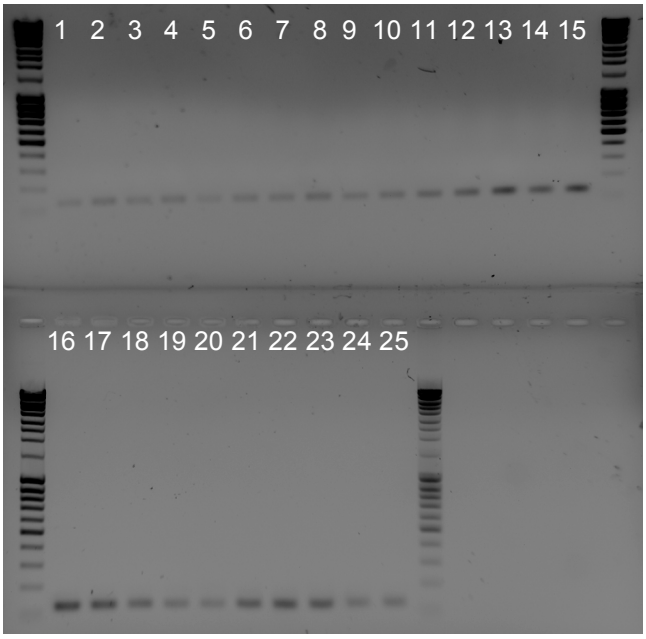
Amounts given in µl account for a 10 nM working stock solution

fw→ ↓r v	0.1µl	0.2µl	0.4µl	0.8µl	1.6µl
0.1µl	1	2	3	4	5
0.2µl	6	7	8	9	10
0.4µl	24	25	10	11	12
0.8µl	20	21	22	23	14
1.6µl	19	18	17	16	15

The most efficient and most sensitive combination of primer concentrations was evaluated to be further used in qPCR. The combination of different primer dilutions used for the assay is listed in table II-2 and a 1% agarose gel showing the result is displayed in figure II-3.

Figure II-3: PCR - titration primer concentration

2.5 µl PCR product/lane



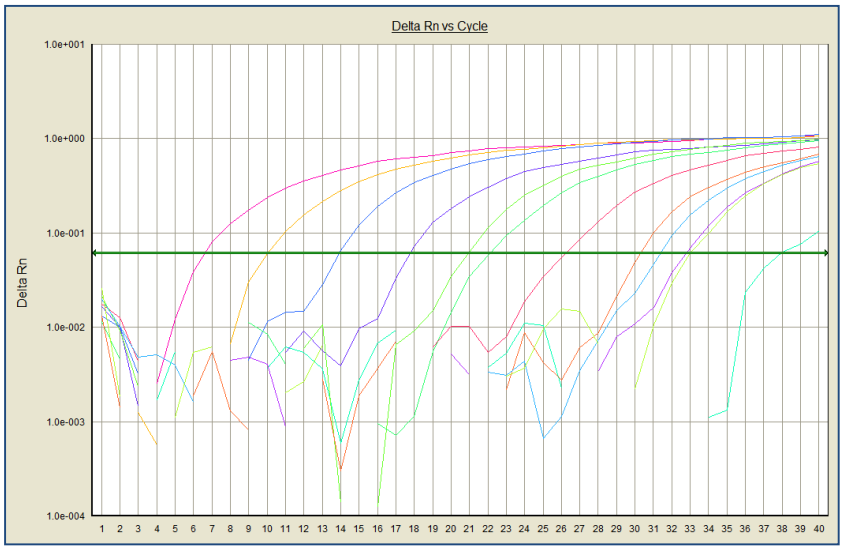
Reactions for quantitative real-time PCR were set up using 10 µl of Perfecta qPCR FastMix with UNG (uracil-N-glycosylase for the enzymatic destruction of contaminants) and low ROX (passive, internal reference dye for the normalization of background fluorescence emission), 2 µM primer SJR#17, 1 µM primer SJR#18, 125 nM probe #SJRb (labeled FAM/TAMRA) and 5 µl template DNA in a total volume of 20 µl per well in 96-well plates. To avoid contamination, master mix was pipetted in a different part of the laboratory than sample template; template for standard curves was handled separately and last. Quantitative real time PCR was run using the 7500 Fast Real-Time PCR System from Applied Biosystems, using the following protocol:

Initial denaturation:		95°C	20 sec
40 cycles	denaturation:	95°C	3 sec
	annealing and elongation:	60°C	30 sec

Standard dilutions of 10-fold dilutions of plasmid pCRII.gB were run in duplicates with each run, together with at least 4 wells with no template control (NTC) = water. A run of standard curves is shown in figure II-4.

Dilutions 10^4 to 10^{10} of the standard were used in duplicates on each plate; the highest dilution of standard, 10^{10} equated 13.3 copies. Samples were run repetitively 2 - 5 times. The threshold was set at 0.2. The determination of cut-off values and further statistical analyses are explained under II-4. and in the respective chapters where results are presented.

Figure II-4: Standard curves with pCRII.gB as template (10-fold dilutions)



3.9. Western blot

Virions were isolated as described under II.2.2. and viral proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE, 10%) (Laemmli, 1970) after denaturation with 100 mM DTT for 5 min at 95°C. Gels were blotted using the semi-dry method (Kyhse-Andersen, 1984) on PVDF membranes. Membranes were blocked for 1 h at room temperature in PBS-T (PBS with 0.05 % Tween20) containing 1% FBS and incubated with anti-PhHV-1 monoclonal antibody (Lebich et al., 1994) (supernatant of hybridoma cells kindly provided by L. Haas, Tierärztliche Hochschule Hannover) diluted 1:100 in PBS-T with 1% FBS for 1 h at room temperature. Secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Rockland) diluted 1:10,000 in PBS-T with 1% FBS was added after washing for 1 h at room temperature. For detection, enhanced chemiluminescence (ECL Plus Detection System) was used according to the manufacturer's instructions.

4. Phylogenetic analyses and statistics

4.1. Phylogenetic analyses

Cloning and sequencing of gB and Pol fragments used for phylogenetic analyses were carried out as explained under II.3.2 and II.3.6.. Vector maps were initially generated using VectorNTI (Invitrogen). Nucleotide sequences of isolates and of reference strains were translated into amino acid sequences using the software VectorNTI. Protein and nucleotide sequences (fasta formate) were aligned using ClustalX multiple alignment tool (www.clustal.org). Phylogenetic analysis using DNA/Protein distance and the Neighbor-joining method (Saitou and Nei, 1987) were processed with PHYLIP (www. evolution.genetics.washington.edu, (Felsenstein, 1989)) and final visual output was generated with the help of FIGTree (www. tree.bio.ed.ac.uk/software/figtree/ (Morariu, 2008)).

4.2. Statistics

Animals considered positive in qPCR were determined as follows. Geometric means and standard deviation (SD) of cycle threshold (C_T) of repeated runs of each sample were calculated and samples with a) no detection in one or more runs, b) a SD of C_T > 1 and c) one or more C_T above 36 were excluded. The coefficient of variation was determined as SD/geometric mean * 100. Further, geometric means and SD of values relative to the standard were calculated. In the phase of establishment of the assay, samples were run in up to six repeats to confirm and reassure reproducibility; later on, samples were run in duplicates.

In order to determine whether data from ELISA assays were positively correlated with SNT values in a linear manner, the Pearson correlation coefficient was defined using Prism5 (www.graphpad.com) as described in III.1.1. and confirmed using the Pearson Correlation (v1.0.3.) of the free statistics software (v1.1.23-r7) of the Office for Research Development and Education (www.wessa.net/rwasp_correlation.wasp/ (Wessa, 2008)). Cut-off values for the ELISA were set as geometric means of a minimum of three control samples proven negative in SNT plus two times their standard deviation. For all dilutions of each sample, this cut-off value was determined and the dilution at which the actual measurement was above the cut-off value was defined as the result.

Confidence intervals (95%) were calculated with the modified Wald method (Agresti A., 1998) via www.graphpad.com.

5. Serological methods

5.1. Serum neutralization test (SNT)

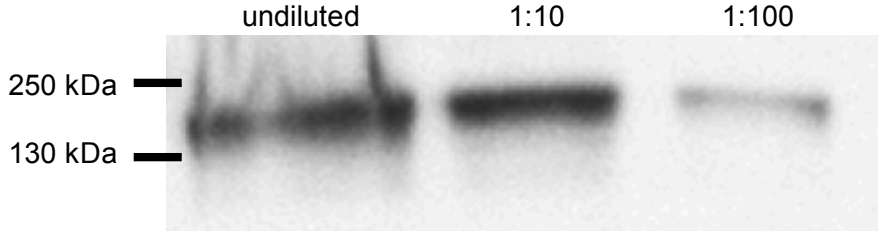
SNTs were carried out in 96-well cell culture plates. Aliquots of 25 µl serum sample were inactivated at 56°C for 30 min and serial two-fold dilution series from 1:4 to 1:256 were made in MEM. Dilutions of serum were incubated with 25 µl PhHV-1 virus suspension (2 x 10^3 pfu/ml) at 37°C for 60 min. CRFK cells in MEM with 5% FBS were added after trypsinizing with a density of 1 x 10^4 cells/well to each sample. Fixation and staining were performed 48 h later with 3% formaline and with 0.05% crystal violet. Virus neutralization was recorded to have occurred, when no cytopathogenic effect of PhHV-1 was visible in wells.

5.2. Enzyme-linked immunosorbent assay (ELISA)

Sera were tested for PhHV-1 in a direct antigen ELISA (Tryland, submitted June 8th 2011). Antigen was prepared growing the reference strain virus isolate at an MOI of 0.1 on CRFK cells for three days. Virions were purified as described under II-2.2. Purified virions were confirmed as PhHV-1 with monoclonal anti-PhHV-1 antibody (Lebich et al., 1994) in western blot analysis as described under II-3.9., shown in figure II-4.

Figure II-4: Detecting of PhHV-1 virions in western blot

Monoclonal anti-PhHV-1 antibody 1:100 in PBS-T 1% FBS



For ELISA, virions were resuspended in coating buffer (0.013M Na_2CO_3; 0.037M $NaHCO_3$; pH 9.5). 96-well plates were coated with antigen at 4°C overnight. After washing the plates with PBST three times, plates were blocked with blocking solution (1X PBS 1 % bovine serum albumine, BSA) for 60 min at room temperature. Seal serum was added in log(2) steps (1:100 – 1:12800) and plates were incubated for 60 min at 37°C. After three wash steps with PBST, peroxidase-conjugated protein A diluted 1:20 000 in blocking solution was added and kept at 37°C for 30 min and washed once before 24 mg/ml substrate 3,3', 5,5' tetramethylbenzidine (TMB) in a 1:1 (v/v) DMSO/EtOH solution, diluted 1:25 in citrate buffer (0.2M citrate; pH 3.95) with 62.5 % H_2O_2, 30%) was added to the wells. The reaction was stopped 15 min later by the addition of 2N H_2SO_4 and absorbances were measured at a

wavelength of 450 nm in an ELISA reader. The ELISA assay was evaluated based on SNTs of sera from the harbor seal population from Svalbard from 2009 (explained in more detail in III.1.1.) and SNT negative and positive samples were run as controls on every plate. Cut-off values were determined as geometric means of three negative control samples plus 2X standard deviation.

Chapter III - RESULTS

1. Impact of PhHV-1 on seals in the Northern hemisphere

Populations of different seal species, harbor seals, harp seals and hooded seals, were tested for PhHV-1 at different locations, Svalbard and the West Ice in the time period from 1998 to 2010. For some of the sample subsets, both, serological and qPCR data was available. For others either swabs or serum samples were not obtained or available for testing.

1.1. PhHV-1 DNA in ocular and nasal swabs of harbor seals, seroprevalences (Svalbard 2009, 2010)

2009

The following section summarizes diagnostic findings in 85 harbor seals captured at the end of August / beginning of September of 2009 on the island Nordøya within the Svalbard archipelago. Sixty-seven percent of all animals captured were female, 33% were male. Ten out of 23 newborn pups were male and 13 female. Four animals were captured during molt, three of them male and one female. No visible and obvious health problems were reported in any of the animals tested.

All 73 nasal swabs were kept in RNAlater (Qiagen, RNA stabilization reagent for tissues) to keep the possibility to later test samples for morbillivirus. DNA extraction was unsuccessful since RNAlater led to the precipitation of one of the buffers in the kit and is known to only conserve larger pieces of tissue and not single cells; the extraction procedure was finished, however. Extracted samples were run in the PhHV-1 gB – specific qPCR assay twice and none of the samples were positive. Since DNA was found in nasal swabs of animals in the following year (kept in normal PBS / antibiotics swab buffer), nasal swabs taken in 2009 are not generally considered negative but no definitive conclusions could be drawn regarding the presence of PhHV-1 DNA.

DNA extracted out of 73 ocular swabs (38% male and 62% female) was tested for PhHV-1 in qPCR in duplicates or triplicates. Due to very low inter-assay SD, two samples with C_T values up to 37.3 were included as well as one sample with very high copy numbers and a SD of C_T of 1.66. In total, six out of 73 samples deemed positive (8%). All animals with PhHV-1 DNA detected in ocular swabs were female, none of them was in molt at the time of capture. Four out of six animals were still pups and the remaining two were only about one year old. No

PhHV-1 DNA could be detected in animals older than one year. Exact animal and qPCR data and values are summarized in table III-1.

Table III-1: qPCR positive samples (ocular swabs) 2009

Geometric means of C_T, standard deviation (SD) of C_T, coefficient of variation (CV) of C_T, geometric means of quantity / DNA copy number and SD of quantity for qPCR positive samples; *direct age determination (tooth) not done for #26 but the BMI value allowed age estimation

animal#	gender	age (years)	mean (C_T)	SD (C_T)	CV (C_T)	mean (quantity)	ELISA titer
26	F	*prob. 1/1+	27,32	1,13	4,14	3904,83	0
31	F	0+	28,38	1,66	5,84	1655,55	1:12800
49	F	1	37,14	0,12	0,33	2,51	1:12800
75	F	0	29,69	0,78	2,63	605,00	0
80	F	0	29,46	1,07	3,63	702,07	n.a.
82	F	0	36,33	0,68	1,87	4,68	n.a.

Sixty-seven sera were tested in SNT, 69 in ELISA (33% males and 67% female animals each). In neutralization assays, 67% of animals were shown to be seropositive, 72% were considered positive in ELISA (correlation shown on p.39). Five out of 21 pups tested serologically showed positive PhHV-1 titers, 16 were negative and for 11 pups no serum was available for testing. Animals in molt were all positive, one with a titer of 1:800 and three with very high titers of 1:12800. Table III-2 summarizes numbers and gender distribution of animals tested and results of serological and molecular tests.

Table III-2: Summary of data 2009

Numbers are total numbers of animals, numbers in parentheses are percentages; numbers in the rows “number of males” and “number of females” are percent of the respective “total number” within the category, percentages in the row “total number” are percent of the total number of animals (n = 85).

2009	total number	number males	number females
Animals tested	85	28 (33)	57 (67)
Serology tested (SNT/ELISA)	67/69 (79/81)	22/23 (33)	45/46 (67)
Ocular swabs tested	73 (86)	28 (38)	45 (62)

2009	total number	number males	number females
SNT positive	45 (67)	15 (33)	30 (67)
ELISA positive	48 (72)	15 (31)	33 (69)
qPCR positive (eye)	6 (8)	0 (0)	6 (100)

Only for four out of six animals positive by qPCR, serum was available for testing. Two had no detectable anti-PhHV-1 antibodies and two were tested with the highest titer of 1:12800 (table III-1).

Correlation ELISA and SNT

The correlation of SNT and ELISA was determined applying the Pearson Product Moment correlation (one-tailed P-value, α-level of 0.05) as a measurement of linear correlation. The correlation coefficient above 0 (r = 0.33) implicates a positive linear correlation between the variables. The correlation is geometrically illustrated in figure III-1.

Figure III-1: Geometrical illustration of the correlation of ELISA and SNT

Graph generated with Pearson-Correlation (v1.0.3) in Free Statistics Software (Wessa, 2008)

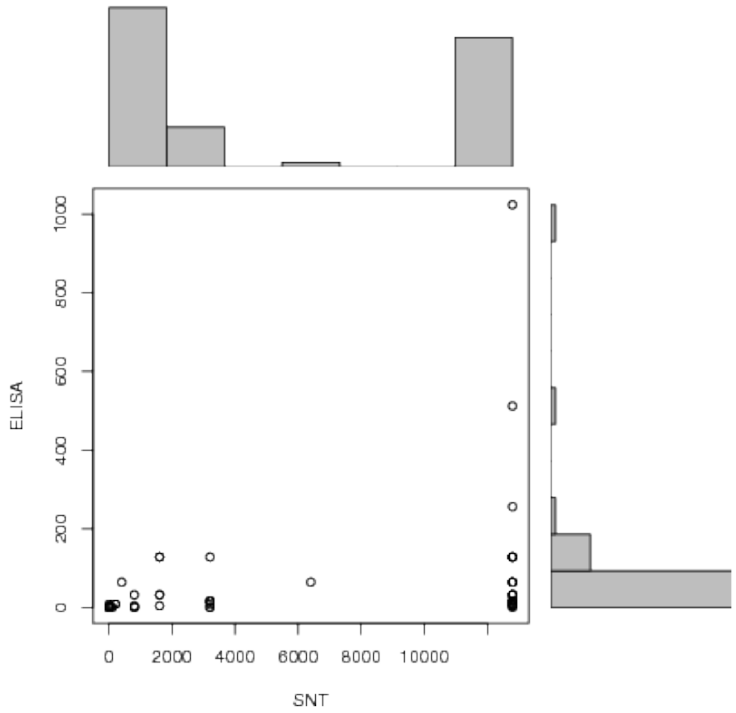


Table III-3: Pearson correlation SNT and ELISA, harbor seals 2009

parameters for the determination of correlation; data generated with the help of PrismX (www.graphpad.com)

Parameter	
Number of XY Pairs	66
Pearson r	0.33
95% confidence interval	0.098 to 0.53
P value (one-tailed)	0.0032
R square	0.11

2010

The following section summarizes results of qPCR and ELISA from animals captured at the end of August / beginning of September 2010. One hundred and two harbor seals were sampled on the islands Sørøya and Midtøya of Svalbard. Forty percent of animals sampled were female and 60 % male. Seven animals were female newborn pups, two were male pups. A total of 21 seals were in molt at the time of sampling, 13 of which were female and 8 male.

Both, ocular and nasal swabs of the data subset of 2010 were available for testing.

As described above, samples with an inter-assay SD of C_T below 1 (run in duplicates) were considered positive and again, a sample above C_T values of 36 was included due to the low SD of C_T (< 0.64) and due to the fact that the same animal was tested positive for PhHV-1 DNA in the nasal swab as well. In total, two out of 102 samples were tested positive for PhHV-1 DNA in eye swabs. One of these two animals was an adult male and one an adult female, both of them were one year old and not in molt. The female harbor seal that was diagnosed with PhHV-1 DNA in the eye was the only animal positive in qPCR testing of nasal swabs. As in 2009, no viral DNA could be found in any of the swabs taken from adult harbor seals. Detailed values and animal data of qPCR positive animals are summarized in table III-4.

Table III-4: qPCR positive samples (ocular and nasal swabs) 2010

Geometric means of C_T, standard deviation (SD) of C_T, coefficient of variation (CV) of C_T, geometric means of quantity / DNA copy number and SD of quantity are given for qPCR positive samples;

swab	animal#	gender	age (years)	mean (C_T)	SD (C_T)	CV (C_T)	mean (quantity)	ELISA titer
ocular	22	M	1	29,87	0,62	2,09	5901,85	0
ocular	32	F	1	39,24	0,10	0,25	16,03	1:1600
nasal	32	F	1	33,25	0,64	1,94	543,10	1:1600

Serological testing of 83 animals (81% of animals captured) revealed an overall prevalence of PhHV-1 in the population of 87% (72 animals) in 2010. Forty-two percent of anti-PhHV-1 antibody positive animals were male, 58% female. An overview of numbers and gender distribution of animals captured in 2010 are shown in table III-5.

Table III-5: Summary data 2010

The table shows numbers of animals tested and the number of positives. Numbers are total numbers of animals, numbers in parentheses are percentages, numbers in the rows "number of males" and "number of females" are percent of the respective "total number" within the category, percentages in the row "total number" are percent of the total number of animals (n = 102).

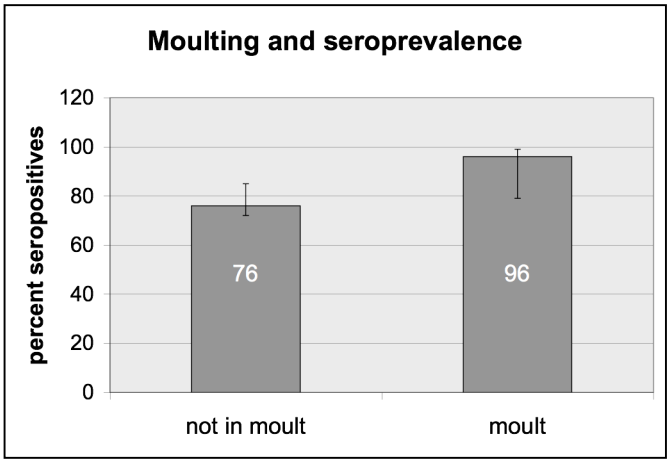
2010	total number	number of males	number of females
Animals tested	102	41 (40)	61 (60)
Serology tested (ELISA)	83 (81)	31 (37)	52 (63)
Ocular swabs tested	102 (100)	41 (40)	61 (60)
Nasal swabs tested	102 (100)	41 (40)	61 (60)
ELISA positive	72 (87)	30 (42)	42 (58)
qPCR positive eye	2 (2)	1 (50)	1 (50)
qPCR positive nose	1 (1)	0 (0)	1 (100)

Out of 21 animals in molt, 20 tested positive for anti-PhHV-1 antibodies with titers varying from 1:400 to 1:12800, generally titers were relatively high. Newborn seal pups had low titers (1:400 to 1:1600) in five cases and four animals were shown seronegative.

Animals in Molt 2009/2010

Figure III-2: Impact of molting on seroprevalences

Numbers of ELISA positive samples of animals in moult and not moulting are compared, 95% confidence intervals are included in the graph. n = 120



Comparison of seroprevalences of PhHV-1 in animals in molt and animals which were not in molt reveals that animals in molt have higher levels of detectable antibodies against PhHV-1 (shown in figure III-2).

Due to the low number of samples of animals in molt (n = 25), data from 2009 and from 2010 were combined. However, only a trend with no statistical significance could be shown.

1.2. Serological studies on a harbor seal population off Svalbard over time

Serum samples were taken from harbor seals captured on the Svalbard archipelago at the beginning of September 1998, at the end of June / beginning of July 1999 and 2000, and at the end of August / early September of 2009 and 2010. Results from 2009 and 2010 were already described in III-1.1. but will be included in this subset in order to complete the picture of seroprevalences in the population over time.

The age distribution of animals captured is illustrated in figure III-3. The graphic shows that the majority of young animals was captured in later years.

Figure III-4 illustrates the gender distribution. In 1998 only male animals were captured and sampled. In all other years, samples from both genders were obtained but more females were caught and sampled always.

Figure III-3: Age distribution

Age of seals captured in 1998 (n=59), 1999 (n=73), 2000 (n=81), 2009 (n= 69) and 2010 (n= 83)

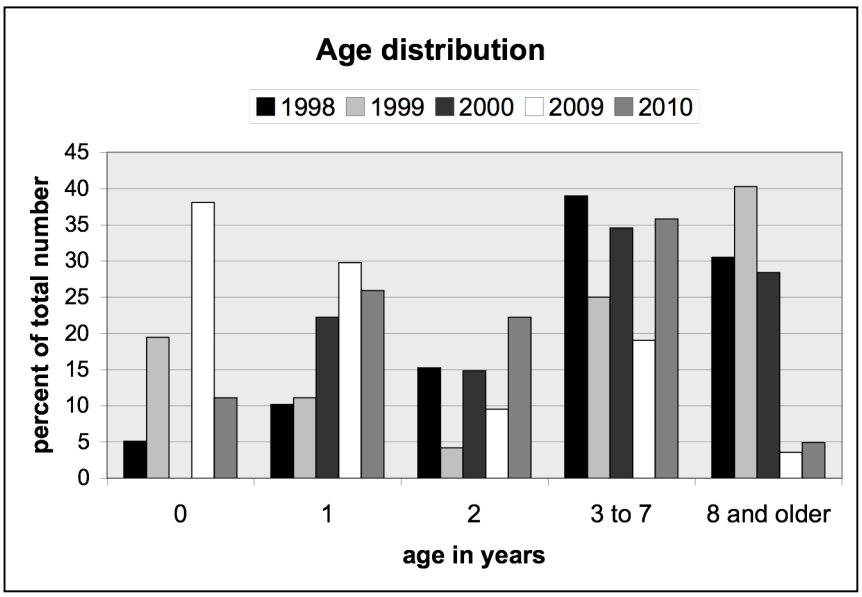
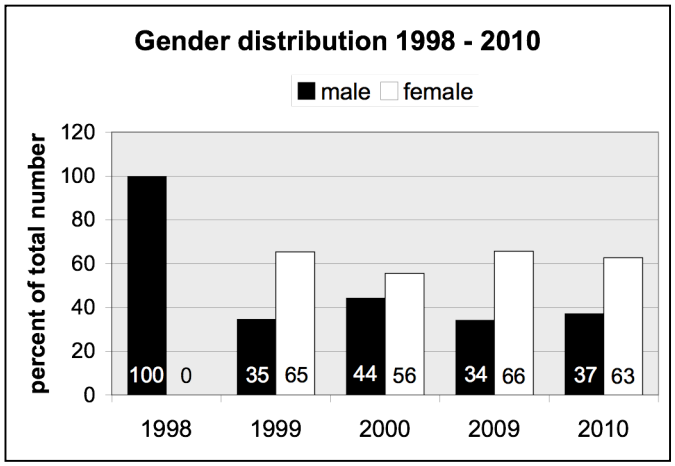


Figure III-4: Gender distribution

Gender of seals captured in 1998 (n=59), 1999 (n=72), 2000 (n=81), 2009 (n= 69) and 2010 (n= 83)

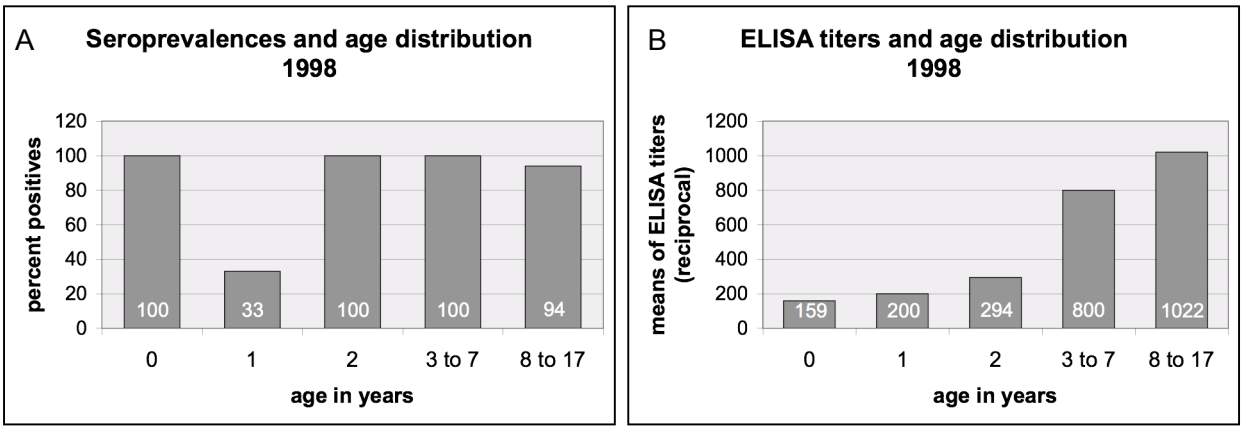


1998

In 1998, blood samples were taken from 59 harbor seals from South Forlandsøy, Kobbebukta and Tvihyrnigen and Krykkjeskjæra. All animals tested were males and three of these were newborn pups. Ninety-two percent of animals were considered seropositive for anti-PhHV-1 antibodies. All samples obtained from pups were positive by ELISA but showed very low titers of 1:100. Analyzing seroprevalence data regarding the age of animals exemplarily for data obtained in 1998, significantly fewer animals were detected with anti-PhHV-1 antibodies at the age of one year (figure III-5A). At the same time, geometric means of ELISA titers, as graphically illustrated in figure III-5B, show that antibody titers rise progressively with age. Geometric means of ELISA titers (reciprocal) were more than 5-fold higher in the animal group of eight years and older than in animals under one year of age.

Figure III-5: Serology and age distribution 1998

A: Seroprevalences in percent of total of different age groups (n = 59)
B: Geometric means of ELISA titers of seropositive animals in different age groups (n = 59)



1999

In 1999, 73 animals were captured in South Forlandsøy, Kobbebukta, Krykkjeskjæra, North of Zieto, Fuglehuken and Forlandet-mid. Harbor seals of both genders were part of the study, including 25 males, 47 females and one animal with undetermined gender. Fourteen animals were newborn pups, 4 of them were males and 10 females. Overall, 88% of animals were seropositive, 34% male and 66% female. Out of 14 pups tested, 6 were seropositive. Seropositivity in neonatal harbor seals was therefore only 31% compared to 100% in weaned and adult animals.

2000

At the end of June / early July 2000, 81 harbor seals one year of age and older were captured and sampled on Sørøya, Midtøya, Forlandet-mid, Tvihyrningen and Fuglehuken. Thirty-six animals were male, 45 female. All animals tested positive for anti-PhHV-1 antibodies in ELISA.

1998-2010

PhHV-1 seroprevalences have, to our knowledge, so far only been measured for populations for a singular time point and not over time. For this study, samples obtained irregularly over a timeframe of 12 years were tested in ELISA. Taken together, harbor seals around Svalbard show generally high seroprevalences with annual deviations. Results over time are graphically displayed in Figure III-6 and summarized in table III-6.

Figure III-6: Seroprevalences over time (1998- 2010)

Numbers on bars give percent of seropositive samples, error bars indicate 95% confidence intervals.
n = 59, 73, 81, 69, 83

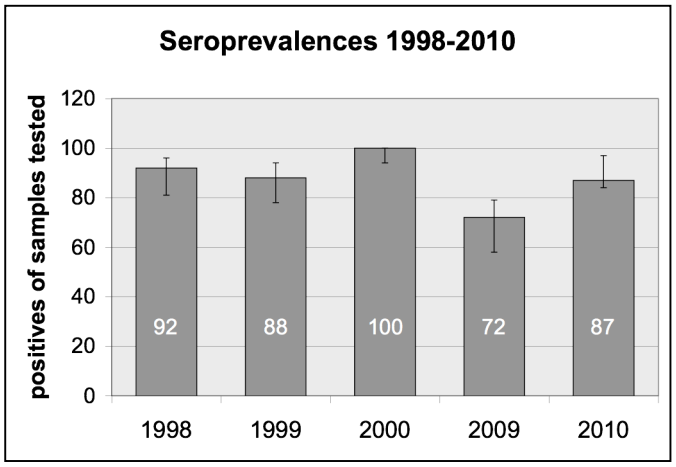


Table III-6: Summary of animal data and serological results 1998-2010

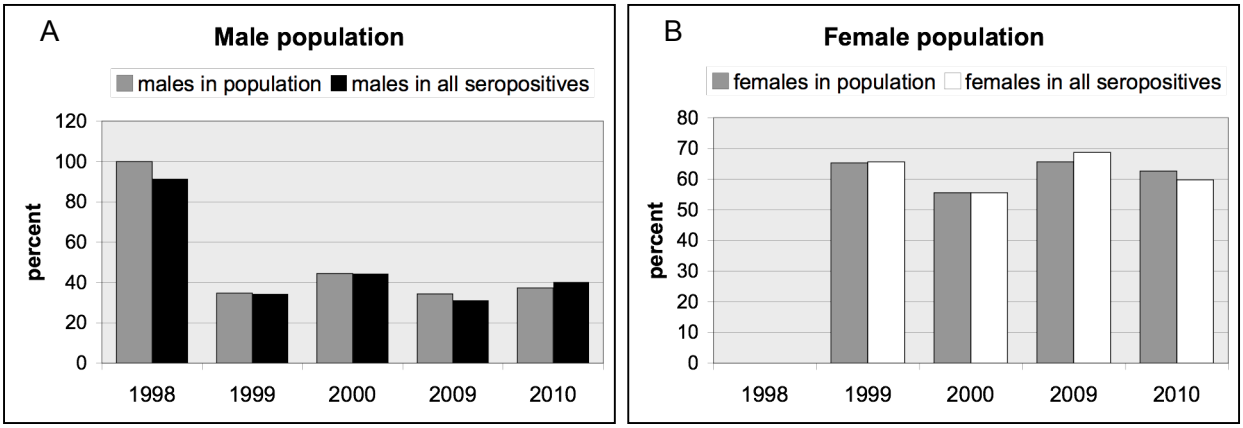
Numbers are either total numbers (n) or % of the population (tested) as indicated in the first column

	1998	1999	2000	2009	2010
number of samples (n)	59	73	81	69	83
(%) gender male/female	100 / 0	35 / 65	44 / 56	33 / 67	40 / 60
pups (<1 year of age) (n)	3	14	0	4	5
molting (n)	n.a.	n.a.	n.a.	4	21
animals ELISA positive (%)	92	88	100	72	87

No gender related differences in seroprevalence could be detected as shown by the comparison of the proportion of positive samples from each gender with total percentages of the respective gender illustrated in figure III-7.

Figure III-7: Seroprevalence and gender

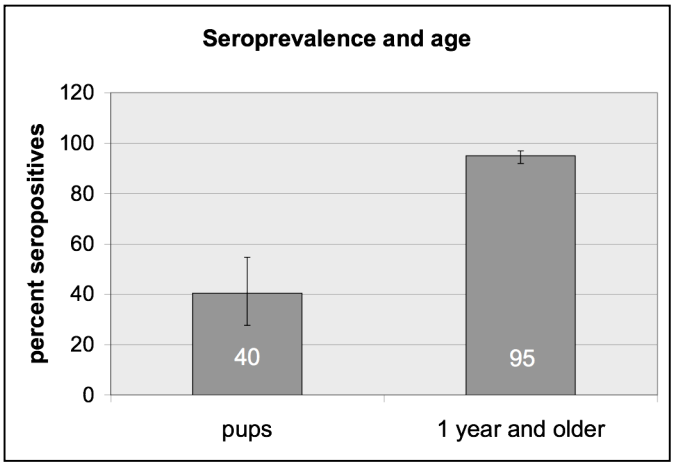
A: Comparison proportion males in the population with percent seropositive males within all positives
n males 1998-2000, 2009, 2010 = 59, 25, 36, 23, 31
B: Comparison proportion females in the population percent seropositive females within all positives
n females 1998-2000, 2009, 2010 = 0, 47, 45, 44, 52



Seroprevalences over time were significantly lower in pups than in adults (figure III-8).

Figure III-8: Seroprevalences in pups and adults

Numbers on bars are percent of all samples tested positive in ELISA; error bars indicate 95% confidence intervals.
n pups = 47, n adults = 316



1.3. PhHV-1 in harp seals and hooded seals (West Ice 2009 / 2010)

2009

DNA extracted from swabs taken from hooded seals and harp seals sampled in the West Ice during an expedition in 2009 were tested by qPCR. All hooded seals were pups between four days and one month of age, harp seals were commercially hunted and of different age. No age data was available for harp seals. In total, 236 hooded seals (116 male, 120 female) and 271 harp seals (101 male, 170 female) were captured,

Not all of the ocular, nasal and genital swabs (n = 54, 110 and 91, respectively) could be tested for PhHV-1 DNA by qPCR due to problems with the DNA extraction procedure. DNA from some of these samples was extracted with a kit by utilizing the 96-well format isolation option but the extraction failed due to insufficient equipment. DNA isolated from 54 ocular (25 hooded seals, 29 harp seals), 83 nasal (39 hooded seals, 44 harp seals) and 44 genital swabs (22 hooded seals, 22 harp seals) was tested for PhHV-1 DNA by qPCR. Analysis revealed prevalences of 24%, 12% and 18% in ocular, nasal and genital swabs respectively. Table III-7 summarizes percent positives and mean quantity for all ocular, nasal and genital swabs in total and for the two different species separately, further demonstrated graphically in figure III-9.

Figure III-9: qPCR swabs

Percent positives in PhHV-1 qPCR of all swabs tested for ocular, nasal and genital swabs with numbers given on bars, 95% confidence intervals are indicated for each subset.
n hooded seal = 25, 39, 22
n harp seal = 29, 44, 22

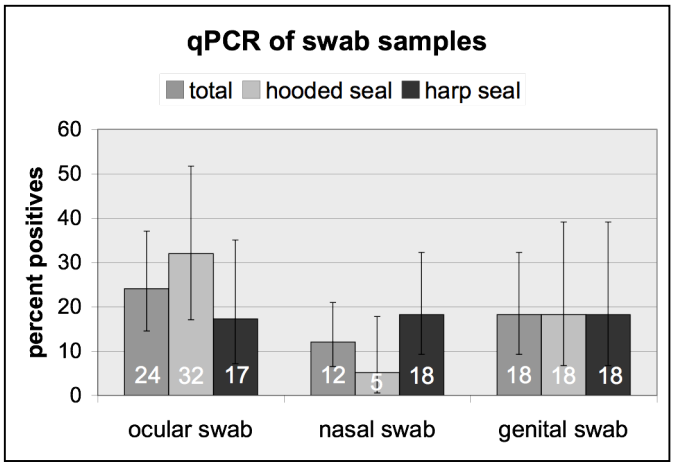


Table III-7: qPCR positive swabs and mean DNA copy numbers

Numbers of positives with percent in parentheses and mean quantities for all animals tested and for the different species

	total	hooded seal	harp seal
ocular swabs			
qPCR positives	13 (24)	8 (32)	5 (17)
DNA copy number (geometric means)	89.0	139.84	43.2
nasal swabs			
qPCR positives	10 (12)	2 (5)	8 (18)
DNA copy number (geometric means)	61.75	24.83	77.55
genital swabs			
qPCR positives	8 (18)	4 (18)	4 (18)
DNA copy number (geometric means)	25.37	16.23	39.66

All samples were tested at least in duplicates; up to six repeats were conducted early on in the establishment of the assay in order to reassure repeatability. Detailed results for qPCR testing of DNA extracted from ocular, nasal and genital swabs divided by species are summarized in table III-8 – III-10.

Table III-8: qPCR results of ocular swab samples

Geometric means of C_T, standard deviations (SD) of C_T, coefficents of variation (CV) of C_T, geometric means of quantities / DNA copy number and standard deviations (SD) of quantities

species	animal#	gender	C_T	SD (C_T)	CV (C_T)	quantity	SD (quantity)
hooded seal	20	female	28,94	0,55	1,92	1661,48	404,01
	86	male	34,09	0,70	2,07	59,56	22,12
	89	female	34,46	0,14	0,40	46,70	6,77
	92	female	35,12	0,28	0,81	55,79	9,74
	123	male	32,35	0,49	1,51	224,40	52,84
	213	female	35,04	0,50	1,43	26,75	8,75
	214	male	27,96	0,48	1,73	2123,91	716,26
	218	female	34,54	0,04	0,11	44,47	1,11
harp seal	192	female	35,20	0,49	1,40	27,44	24,59
	209	male	34,46	0,07	0,19	83,72	3,45
	291	male	35,01	0,17	0,49	30,22	3,42
	292	male	33,16	0,36	1,08	77,72	28,92
	318	female	34,24	0,24	0,71	27,88	4,69

Table III-9: qPCR results of nasal swab samples

species	animal#	gender	C_T	SD (C_T)	CV (C_T)	quantity	SD (quantity)
hooded seal	23	male	33,82	0,16	0,46	40,19	4,20
	309	female	35,25	0,54	1,53	15,34	4,75
harp seal	190	female	31,04	0,14	0,46	254,51	23,45
	229	male	33,25	0,45	1,37	57,16	18,10
	257	female	35,80	0,26	0,74	5,31	6,82
	274	male	31,84	0,28	0,87	144,89	25,12
	307	female	33,10	0,10	0,32	62,87	4,53
	323	female	34,65	0,82	2,36	22,59	11,05
	334	male	33,04	0,21	0,63	65,40	8,49
	475	male	28,55	0,15	0,53	1257,98	125,87

Table III-10: qPCR results of genital swab samples

species	animal#	gender	C_T	SD (C_T)	CV (C_T)	quantity	SD (quantity)
hooded seal	86	male	35,67	0,35	0,99	8,72	4,05
	123	male	34,57	0,14	0,40	18,44	4,97
	223	male	33,40	0,23	0,69	40,78	7,92
	281	female	35,39	0,19	0,53	10,60	3,05
harp seal	200	male	34,85	0,66	1,89	15,22	10,78
	202	female	30,79	0,20	0,64	235,87	69,33
	251	male	32,85	0,31	0,95	58,53	26,12
	264	male	35,22	0,38	1,08	11,78	5,83

Complete data sets were not available for all samples. Only two animals were found to have PhHV-1 DNA detectable in more than one swab: animal #86 and #123. Both were hooded seals tested positive in ocular and genital swab, nasal swab samples were not available for either of them. For animals found with high DNA copy numbers in ocular swabs - such as animal #20 or #214 (hooded seals) - no nasal swab samples were available. Similarly, no ocular swabs were available for testing for animals tested positive in nasal swabs. Geometric means of DNA copy numbers / quantities as summarized in table III-11 were comparable in different swab samples with a trend to be lower in genital swabs. Due to the inhomogeneous groups with different species and too few sample numbers, statistical analysis was difficult to perform and therefore data is presented descriptively. Serological testing was carried out on few samples (from selected qPCR positive and negative animals) by SNT, which revealed no detectable PhHV-1 antibodies. No ELISA testing was done on serum samples.

DNA extracted from tissue samples of liver, lung, kidney, spleen and reproductive organs was tested in qPCR and all samples were negative.

Virus isolation was attempted on CRFK cells, inoculating cells seeded in 24-well plates with 50 µl of swab sample. Cytopathogenic effects were observed upon initial inoculation and the first passages but could not be confirmed in further passages.

Table III-11: qPCR results of ocular, nasal and genital swabs

animal #	ocular swab	nasal swab	genital swab	species	gender
20	1661,48	n.a.	n.a.	hooded seal	female
23	n.a.	40,19	n.a.	hooded seal	male
86	59,56	n.a.	8,72	hooded seal	male
89	46,70	neg	n.a.	hooded seal	female
92	55,79	neg.	n.a.	hooded seal	female
123	224,40	n.a.	18,44	hooded seal	male
190	n.a.	254,51	n.a.	harp seal	female
192	27,44	neg.	neg.	harp seal	female
200	neg.	neg.	15,22	harp seal	male
202	n.a.	neg.	235,87	harp seal	female
209	83,72	n.a.	n.a.	harp seal	male
213	26,75	neg.	n.a.	hooded seal	female
214	2123,91	n.a.	neg.	hooded seal	male
218	44,47	neg.	n.a.	hooded seal	female
223	n.a.	neg.	40,78	hooded seal	male
229	n.a.	57,16	n.a.	harp seal	male
251	n.a.	n.a.	58,53	harp seal	male
257	n.a.	5,31	n.a.	harp seal	female
264	n.a.	n.a.	11,78	harp seal	male
274	n.a.	144,89	n.a.	harp seal	male
281	n.a.	neg.	10,60	hooded seal	female
291	30,22	n.a.	n.a.	harp seal	male
292	77,72	n.a.	n.a.	harp seal	male
307	neg.	62,87	n.a.	harp seal	female
309	n.a.	15,34	n.a.	hooded seal	female
318	27,88	n.a.	n.a.	harp seal	female
323	n.a.	22,59	n.a.	harp seal	female
334	n.a.	65,40	n.a.	harp seal	male
475	n.a.	1257,98	n.a.	harp seal	male

2010

DNA extracted from 28 ocular and 27 nasal swabs (55 swabs in total) from 29 animals (26 animals rendered both, ocular and nasal swabs) were tested by qPCR for presence of PhHV-1 DNA. Of the 29 tested animals 9 were harp seals (5 females and 4 males) and 20 were hooded seals (12 were females and 8 males). Age data was available only for female hooded seals, which were between one and seven years of age. All samples tested negative for PhHV-1 DNA. All swab samples had been stored at -20°C.

2. Impact of PhHV-1 on seals in the Southern hemisphere

Various species of seals inhabiting Antarctica were tested for seroprevalences of anti-PhHV-1 antibodies during different expeditions in 2000 - 2002 and in 2010. Reports on PhHV-1 seroprevalence in seals from the Southern hemisphere are so far scarce.

2.1. Serological studies on pinnipeds captured in Antarctica in 2000-2001

Blood samples were obtained from 88 Antarctic fur seals in Bouvetøya and from 20 Ross seals, 20 Weddell seals and 9 crabeater seals in King Haakon VII Sea, off Queen Maud Land. Results of serological testing for all species are presented in table III-12.

Table III-12: Seroprevalences seal spp. from Antarctica 2000 / 2001

Table adapted from manuscript (Tryland, submitted June 8th 2011)

species	PhHV-1
Antarctic fur seals: adult females	25/43 (58)
Antarctic fur seals: adult males	4/6 (67)
Antarctic fur seals: pups	13/25 (39)
Antarctic fur seals (total)	42/74 (57)
Weddell seals	20/20 (100)
Ross seals	3/20 (15)
crabeater seals	4/9 (44)

Atlantic fur seals showed seroprevalences of 57%. Twenty-six percent of animals were male, 74% female. In one pup the gender could not be determined. A 10% difference in seroprevalence between male and female adults was not significant and most likely resulted

from the small number of samples obtained for bulls. Interestingly, overall seroprevalence as well as titers showed the trend to be higher in pups than in adults, a finding that was not significant, however (figure III-10A and III-10B).

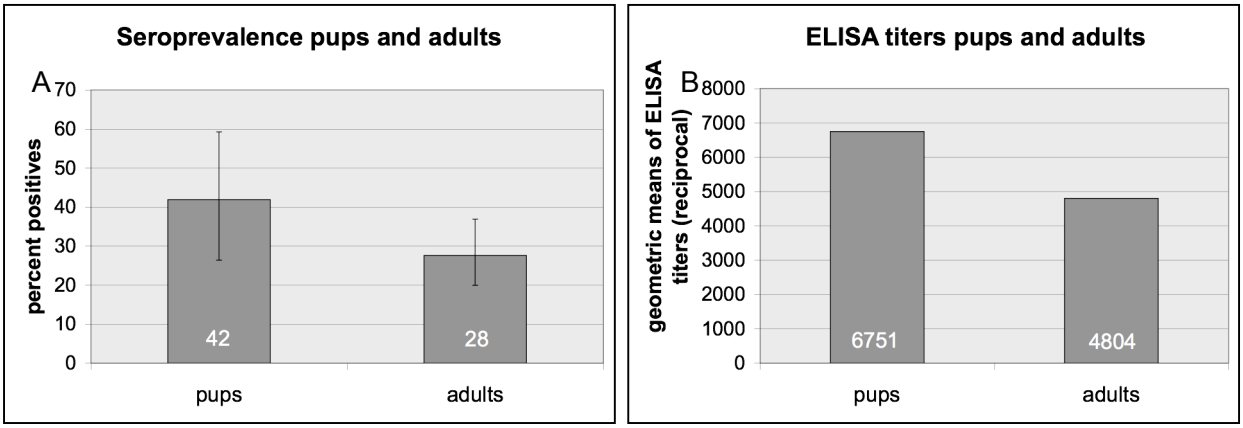
All Weddell seals tested were highly (geometric means of titers (reciprocal) = 8063) positive for anti-PhHV-1 antibodies.

The lowest seroprevalence in the species sampled were seen in Ross seals with three out of 20 animals having anti-PhHV-1 antibodies as determined by ELISA.

The very small sample number of crabeater seals (n = 9) did not allow conclusions for the entire population, but 4 out of 9 animals showed high titers of 1:6400 (n = 2) and 1:12800 (n = 2).

Figure III-10: Seroprevalences in pups and adults, Antactic fur seals 2000 - 2001

Numbers on bars are given in percent, error bars in A indicate 95% confidence intervals
n adults = 29, n pups = 13



2.2. PhHV-1 in pinnipeds captured in Antarctica in 2010

A group of scientists from the University of Madrid, Spain, captured Antarctic fur seals, Weddell seals and Southern elephant seals during a research expedition in 2010. They obtained blood samples from most animals of all species (n = 51, n = 15 and n = 4 respectively). Nasal swabs were obtained from all 80 animals. Animals were sampled at three different locations, as listed in table III-13.

DNA was extracted from swab samples by the group in the laboratory in Spain using the blood and tissue kit (Qiagen) according to the manufacturer's instructions. None of the DNA samples extracted from nasal swabs tested positive for PhHV-1 by qPCR in duplicates.

All Antarctic fur seals captured in two of the three locations were male. In 14% of the samples, anti-PhHV-1 antibodies could be detected with titers between 1:1600 and 1:6400.

Eleven out of 17 Weddell seals were positive for PhHV-1 with titers in the same range as seen for Antarctic fur seals. The discrepancy between seroprevalence in males and females (78% and 50% respectively) is likely accounted by low sample numbers (male n = 9 and female n = 8).

Table III-13: Animal numbers and location Antarctica 2010

animal species	total blood/serum	location	samples blood/serum
Antarctic fur seal	58/51	Avian Island	14/14
		Deception Island	44/37
Weddell seal	17/15	Rongé Island	1/1
		Avian Island	2/2
		Deception Island	14/12
Southern elephant seal	5/4	Avian Island	4/3
		Deception Island	1/1

Table III-14: Seroprevalences Antarctica 2010

species	PhHV positive totals	PhHV positive males	PhHV positive females	PhHV pos. Avian Island	PhHV pos. Deception Island	PhHV pos. Rongé Island
Antarctic fur seal	8/58 (13.8)	8/58 (13.8)	0/0 (0)	6/14 (42.9)	2/44 (4.5)	-
Weddell seal	11/17 (64.7)	7/9 (77.8)	4/8 (50)	1/2 (50)	9/14 (64.3)	0/1 (0)
Southern elephant seal	4/5 (80.0)	1/2 (50)	3/3 (100)	4/4 (100)	0/1 (0)	-
total	23/80 (28.8)	16/69 (23.2)	7/11 (63.6)	11/20 (55)	11/59 (18.6)	0

Southern elephant seals have, to our knowledge so far never been tested for PhHV-1 seroprevalence in the wild. Four out of five animals captured in two of the three locations were ELISA-positive, with high titers of 1:3200 (n = 2) and 1:12800 (n = 2). Exact numbers for different species, gender and capture location are summarized in table III-14.

Only small sample numbers were obtained from each location. One Southern elephant seal was captured on Rongé Island and shown to be negative for anti-PhHV-1 antibodies, whereas Southern elephant seals from Avian Island and from Deception Island (n = 2 each) were all highly seropositive.

3. Phylogenetic comparison of PhHV-1 gB and Pol sequences

Phylogenetic analyses revealed that PhHV-1 is grouped in a cluster of alphaherpesviruses from terrestrial carnivores (canid: CaHV-1 and felid: FeHV-1). As described, partial sequences of glycoprotein B (gB) and DNA polymerase (Pol) were TA cloned and sequenced. Sequences obtained from several readouts were 470 bp (gB) and 500 bp (Pol) in length. Alignments (clustalX, VectorNTI, Invitrogen) of the sequences available in GenBank are shown in figures III-11 and III-12.

For gB, nucleotide sequences of two isolates from European waters (S81228 and Z68147) and of one isolate from the Pacific (U92270) were available for comparison. The partial gB sequence showed nucleotide differences between European and Pacific isolates in 21 out of 470 positions (4.47%), as shown in blue. Only one was a non-synonymous nucleotide substitution (methionine - isoleucine). Two SNPs (C-T) were found in which the reference strain, although originally isolated from an animal in the North Sea, was similar to the Pacific strain and differed from the other Atlantic isolates (indicated in green). One was synonymous and one nucleotide substitution changed the amino acid sequence from glycine to alanine.

Partial gB sequences amplified by conventional PCR from PhHV-1 positive ocular samples from harbor seals as described in III-1.1. were sequenced and revealed one silent SNP (prolin to prolin). The position of the mutation (C to T) is indicated in pink in Figure III-1.1., the site of mutation is different from other sites of mutation seen for differences between Pacific and Atlantic strains.

Figure III-11: Alignment of gB nucleotide sequences

PhHVgB_isolate is the reference strain used for this study, for others, GenBank accession numbers are given, sequences were aligned with VectorNTIalign

```
451                                                  500
PhHVgB_isolate      (1) ----------------------------------------------AGCTC
 PhHVgB_S81228      (1) ----------------------------------------------AGCTC
 PhHVgB_U92270      (1) ---------------------------ACAACTGTATGGTCTGGAAGCTC
 PhHVgB_Z68147    (451) AATATTTACTATAAGAATATTATTATAACAACTGTATGGTCTGGAAGCTC
                        501                                          550
PhHVgB_isolate      (6) GTATGCCGTAGTCACTAACATGCATACTGATAGAGTACCTATAAAGGTTC
 PhHVgB_S81228      (6) GTATGCCGTAGTCACTAACATGCATACTGATAGAGTACCTATAAAGGTTC
 PhHVgB_U92270     (24) GTATGCTGTAGTTACCAACATGCATACTGATAGAGTTCCTATAAAGGTTC
 PhHVgB_Z68147    (501) GTATGCCGTAGTCACTAACATGCATACTGATAGAGTACCTATAAAGGTTC
                        551                                          600
PhHVgB_isolate     (56) AAGAAATTACAGAATTGATTGATCGTAGGGGTATGTGCCTCTCAAAGGCT
 PhHVgB_S81228     (56) AAGAAATTACAGAATTGATCGATCGTAGGGGTATGTGCCTCTCAAAGGCT
 PhHVgB_U92270     (74) AAGAAATTACAGAATTGATTGACCGAAGGGGTATGTGCCTCTCAAAGGCT
 PhHVgB_Z68147    (551) AAGAAATTACAGAATTGATCGATCGTAGGGGTATGTGCCTCTCAAAGGCT
                        601                                          650
PhHVgB_isolate    (106) GATTATATTCGCAATAATTACGAGTTTACTGCATTTGATAAAGATGAAGA
 PhHVgB_S81228    (106) GATTATATTCGCAATAATTACGAGTTTACTGCATTTGATAAAGATGAAGA
 PhHVgB_U92270    (124) GATTATATTCGCAACAATTACGAGTTTACCGCATTTGATAAAGACGAAGA
 PhHVgB_Z68147    (601) GATTATATTCGCAATAATTACGAGTTTACTGCATTTGATAAAGATGAAGA
                        651                                          700
PhHVgB_isolate    (156) CCCCAGAGAAATGCATTTAAAACCCTCAAAATTTAATACACCCGGTTCTC
 PhHVgB_S81228    (156) CCCCAGAGAAATGCATTTAAAACCCTCAAAATTTAATACACCCGGTTCTC
 PhHVgB_U92270    (174) CCCCAGAGAGATGCATTTAAAACCCTCAAAATTTAATACACCTGGTTCTC
 PhHVgB_Z68147    (651) CCCCAGAGAAATGCATTTAAAACCCTCAAAATTTAATACACCCGGTTCTC
                        701                                          750
PhHVgB_isolate    (206) GTGGATGGCATACGACAAATGATACGTATACAAAAATTGGGAGTCCTGGT
 PhHVgB_S81228    (206) GTGGATGGCATACGACAAATGATACGTATACAAAAATTGGGAGTCCTGGT
 PhHVgB_U92270    (224) GTGGATGGCATACGACAAATGATACATATACAAAAATTGGAAGTCCTGGG
 PhHVgB_Z68147    (701) GTGGATGGCATACGACAAATGATACGTATACAAAAATTGGGAGTCCTGGT
                        751                                          800
PhHVgB_isolate    (256) TTTTATCGTACGGGAACATCTGTAAATTGTATTGTCGAAGAAGTTGATGC
 PhHVgB_S81228    (256) TTTTATCGTACGGGAACATCTGTAAATTGTATTGTCGAAGAAGTTGATGC
 PhHVgB_U92270    (274) TTTTATCGTACGGGAACATCTGTAAATTGTATTGTTGAAGAAGTTGATGC
 PhHVgB_Z68147    (751) TTTTATCGTACGGGAACATCTGTAAATTGTATTGTCGAAGAAGTTGATGC
                        801                                          850
PhHVgB_isolate    (306) CAGATCTGTATATCCATATGATTCCTTTGCCATTTCAACTGGAGATATAA
 PhHVgB_S81228    (306) CAGATCTGTATATCCATATGATTCCTTTGGCATTTCAACTGGAGATATAA
 PhHVgB_U92270    (324) CAGATCTGTATATCCATATGATTCTTTGCTATTTCAACTGGAGATATAA
 PhHVgB_Z68147    (801) CAGATCTGTATATCCATATGATTCCTTTGGCATTTCAACTGGAGATATAA
                        851                                          900
PhHVgB_isolate    (356) TTCATATGTCTCCATTTTTTGGTTTACGTGATGGAGCTCATACAGAACAT
 PhHVgB_S81228    (356) TTCATATGTCTCCATTTTTTGGTTTACGTGATGGAGCTCATACAGAACAT
 PhHVgB_U92270    (374) TTCACATTTCTCCATTTTTTGGTTTACGTGATGGAGCTCATACAGAACAT
 PhHVgB_Z68147    (851) TTCATATGTCTCCATTTTTTGGTTTACGTGATGGAGCTCATACAGAACAT
                        901                                          950
PhHVgB_isolate    (406) ACTAGCTATTCAAATGATCGATTTCAACAAATTGAGGGTTATTATCCTAT
 PhHVgB_S81228    (406) ACTAGCTATTCAAATGATCGATTTCAACAAATTGAGGGTTATTATCCTAT
 PhHVgB_U92270    (424) ACTAGCTATTCGAATGATCGATTCCAACAAATTGAGGGTTATTATCCTAT
 PhHVgB_Z68147    (901) ACTAGCTATTCAAATGATCGATTTCAACAAATTGAGGGTTATTATCCTAT
                        951                                         1000
PhHVgB_isolate    (456) TGATTTGGATACCA-----------------------------------
 PhHVgB_S81228    (456) TGATTTGGATACCAGACTACAAGTTGGGGGACCAGTTTCCAGAAA-----
 PhHVgB_U92270    (474) TGATTTGGATACCAGACTAAAAGTTGGAGCACCAGTTTCTAGAAACTTTC
 PhHVgB_Z68147    (951) TGATTTGGATACCAGACTACAAGTTGGGGGACCAGTTTCCAGAAACTTTC
                        1001                                        1050
```

Figure III-12: Alignment of DNA dependent DNA polymerase nucleotide sequences

PhHVpol_isolate is the reference strain used for this study, for the other sequence, the respective GenBank accession number is given, sequences were aligned with VectorNTIalign

```
1                                                  50
                          801                                                850
PhHVpol_isolate      (1)  ---------------------------------GATATTGGAGATGTTCC
 PhHVpol_U92269    (801)  TTTATCCTCCCAGATAATAGAAATAAACTGGGGGATATTGGAGATGTTCC
                          851                                                900
PhHVpol_isolate     (18)  AGAATCTATGGATTGCGATTTAGTGTTTGATGAAGACTCACAAATGGATG
 PhHVpol_U92269    (851)  AGAATCTGTTGATTGTGATTTAGTGTTTGATGAAGACTCACAAACAGATG
                          901                                                950
PhHVpol_isolate     (68)  ATGGTGAAACTGTTCACGAACCGAGAGCATGTAAAACCAACTCTGGGCGC
 PhHVpol_U92269    (901)  ATATTGAAAATATTCATGAACCAACAGCGTGTAAAACTAACTCTGGGCGC
                          951                                               1000
PhHVpol_isolate    (118)  TCTGTTGGATATCAGGGGGCTAAGGTTTTAGACCCAATTTCTGGATTCCA
 PhHVpol_U92269    (951)  TCTGTTGGATGCCAGGGGGCTAAAGTTTTAGATCCAATTTCTGGATTTCA
                         1001                                               1050
PhHVpol_isolate    (168)  TGTCGACCCGGTTATGGTACTAGATTTTGCTAGCTTATATCCTAGTATAA
 PhHVpol_U92269   (1001)  TGTTGACCCAGTCATGGTACTTGATTTTGCTAGCTTATATCCTAGTATAA
                         1051                                               1100
PhHVpol_isolate    (218)  TCCAAGCTCATAACTTATGTTTTACAACTCTGACAACAAACCCATCCAGT
 PhHVpol_U92269   (1051)  TCCAAGCTCATAACTTATGTTTAACAACTCTAACAACAAACCCATCTAGC
                         1101                                               1150
PhHVpol_isolate    (268)  GTATCTCATCT-AAAAAAGGACATTGATTACTCAGAGTTTATAGTTAACG
 PhHVpol_U92269   (1101)  GTG-CTCATCTTAAAAAAGGACATTGATTACTCAGAGTTTATAGTTAACA
                         1151                                               1200
PhHVpol_isolate    (317)  GCCAAAAGTTAATTTTTGTTCATTCGCATATACGTGAAAGCTTGCTCAGT
 PhHVpol_U92269   (1150)  GTCAAAAGCTAATTTTTGTTCATTCGCATATACGCGAAAGCTTACTCAGC
                         1201                                               1250
PhHVpol_isolate    (367)  ATATTACTTAGGGACTGGCTTGTTATGAGAAAGGCCATTCGAGCGCGTAT
 PhHVpol_U92269   (1200)  ATACTACTTAGAGATTGGCTTGTTATGAGAAAGGCCATTCGAGCGCGTAT
                         1251                                               1300
PhHVpol_isolate    (417)  ACCAAAAAGCTCAGAAGATGAAGCTGTGTTACTAGATAAGCAGCAAGCAG
 PhHVpol_U92269   (1250)  ACCAAAAAGCTCAGAAGACGAAGCTGTGTTACTAGACAAGCAACAAGCAG
                         1301                                               1350
PhHVpol_isolate    (467)  CCATCAAAGTGGTTTGCAACTCAGTGTATGGGTTTACAGGAGTTTCCAAT
 PhHVpol_U92269   (1300)  CGATAAAAGTTGTTTGTAACTCAGTATATGGATTTACAGGAGTTTCCAAT
                         1351                                               1400
PhHVpol_isolate    (517)  GGT-------------------------------------------------
 PhHVpol_U92269   (1350)  GGTTTGTTACCCTGTTTGCACATAGCCGCAACTGTAACAACTATAGGAAG
                         1401                                               1450
```

Only one partial nucleotide sequence of Pol, from an animal from Pacific waters, was available for comparison at the time. The nucleotide sequence differed from the reference strain sequence in 48 positions (9.6 %). Thirty-two substitutions were non-synonymous. In four codons, two nucleotides were different. A frame-shift over three codons changed two out of the three amino acids (serine-histidine-leucine to leucine-isoleucine-leucine).

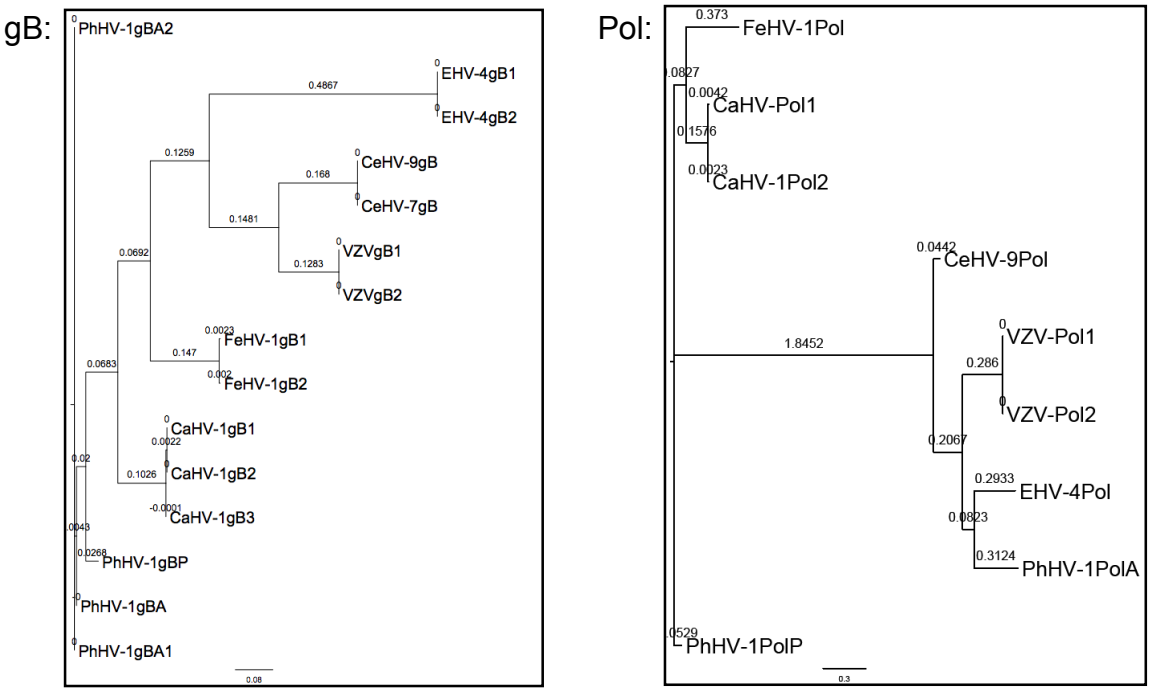
3.1. Phylogenetic tree, nucleotide level

Sequence identity of gB was, as described above, found to be high ranging between 95 and 99% compared to 90% identity for the DNA polymerase gene. Partial sequences of gB and

Pol were phylogenetically analyzed and are shown in relation to other animal varicelloviruses (Fig. III-13). For gB, the Pacific isolate showed greatest distance but all PhHV-1 sequences were seen to be closely related and highly similar to feline and canine gB sequences. Analyses of partial Pol sequences of the reference strain and of one on GenBank available, Pacific, sequence revealed, that the sequence obtained from our reference strain was highly dissimilar and intriguingly clustered with equine herpesvirus type 4 sequences, whilst the Pacific isolate showed the same clustering with feline and canine sequences as observed for gB.

Figure III-13: PhHV-1 gB and polymerase nucleotide sequences

Comparison with homologous sequences from other animal herpesviruses, Neighbor joining method;
gB: A: reference isolate, A1: gbZ68147; A2: gbS81228; A: reference isolate, P: gbU92270
Pol: A: reference isolate, P: gbU92269



3.2. Phylogenetic tree, amino acid level

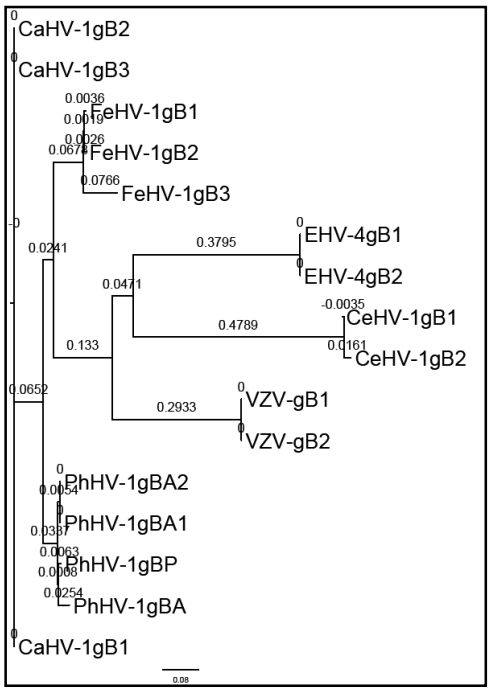
Translated partial gB sequences of the reference strain were compared to available protein sequences (NCBI GenBank), two from Atlantic isolates and one from a virus isolated in the Pacific. Analyses revealed 99% sequence identity. The reference strain, as seen on nucleotide level, clustered with other PhHV-1 sequences and could be shown to be closely related to CaHV-1 and FeHV-1 gB sequences. A comparison of the translated partial Pol sequences of the reference strain with one Pacific amino acid sequence available on NCBI

GenBank on amino-acid level revealed 93% identity. In phylogenetic analyses the translated sequence was shown to be closest related to the Pacific PhHV-1 Pol sequence.

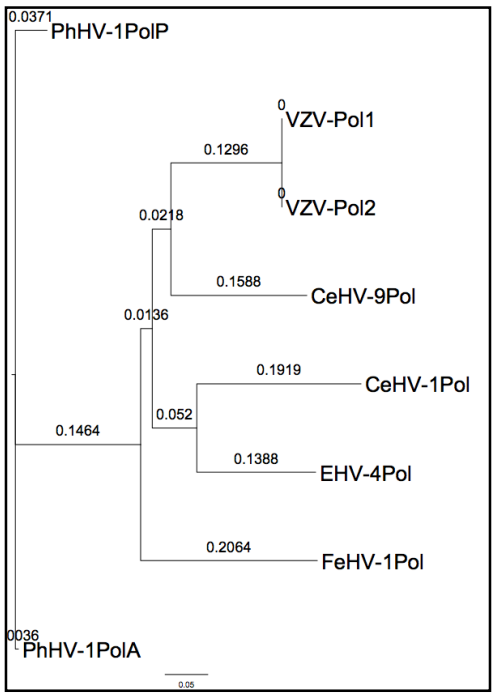
Figure III-14: PhHV-1 gB and polymerase amino acid sequences

Comparison with homologous sequences from other animal herpesviruses, Neighbor joining method; gB: A: reference isolate, A1: gbCAA92272; A2: gbAAP32233; A: reference isolate, P: gbAAB93519 Pol: A: referene isolate, P: gbAAB93518

gB:



Pol:



Chapter IV – DISCUSSION

Little is known about infectious diseases in different seal populations worldwide. PhHV-1 has been studied for several decades but despite several studies of mainly neonatal animals with primary infections in rehabilitation and some snap-shots of serological testing not much has been reported yet.

Results of this study present data on PhHV-1 seroprevalence in various environments, Arctic and Antarctic. Different species inhabiting various habitats worldwide were monitored, some over a long time frame, some only in a singular testing event. Furthermore, a sensitive qPCR assay was established that allows the detection of herpesvirus DNA in ocular, nasal and genital swabs. This is, to our knowledge, the first time that swab samples from wild animals in their natural habitat were tested for PhHV-1 DNA. Vertical transmission was proposed before (Goldstein et al., 2004) and the detection of PhHV-1 DNA in genital swabs in a wild population may suggest that PhHV-1 contributes to abortion events. The hypothesis, that PhHV-1 has a reactivation site in the eye and that the virus may be involved in the etiology of anterior segment ocular disease could not be confirmed since DNA was not detected in ocular swabs from animals older than one year of age. Whether the involvement of PhHV-1 in ocular disease can be completely excluded will be discussed in detail later. Serological monitoring of various populations and the (irregular) follow-up in one population of harbor seals over a period of 12 years contributes to the knowledge about PhHV-1 prevalences worldwide. Work on wildlife in general and multifactorally influenced infectious diseases in particular is difficult and in the following sections, results obtained from the studies will be discussed in this regard.

1. General problems and weaknesses

1.1. Establishment of qPCR

At the time that the qPCR was established, it was not clear that samples other than from Arctic waters would be tested later. The PCR was developed to be as sensitive and as specific as possible, and primers and probe were, as described in II.3.8., designed to bind conserved regions based on an alignment of gB sequences of the isolate used as reference virus and the published partial gB sequences. Later attempts of testing samples from ontarids were therefore not considered at the time when primers and probe were chosen. The qPCR is hence only a reliable tool for testing of samples from phocids in Arctic and

temperate Antlantic, potentially Pacific, locations. The sensitivity of the assay was generally high with a detection limit of 13.3 copies. Whether low virus yields in latent stages and during times of low replication levels can be detected in swabs and tissues, however, cannot be fully assessed and we cannot draw final conclusions that animals tested negative for PhHV-1 in the assay are indeed virus-free.

During the establishment of the assay, high contamination risk due to high sensitivity of the method was experienced, when plasmid DNA extractions and qPCR were conducted in the same room. Through a strict management, separating working steps in different rooms and with different sets of equipment and including large numbers of NTC-wells per plate, it was possible to solve the problem.

Once the method was established and initial problems were overcome, the qPCR did not pose problems anymore. To keep inter-assay variance low, standard dilutions were made on a large scale and kept at -20°C until further use.

1.2. General difficulties with sampling and sample maintenance

Obtaining samples from seals poses a variety of difficulties. Even from animals in captivity it is difficult to acquire swabs, particularly from the eye. The anatomical features and behavior of a non-domesticated carnivore species make the handling of animals difficult and only few seals tolerate medical interventions after long periods of training without sedation. Body shape and the fact that seals can turn their body “within their blubber layer” pose additional difficulties to capture, handling and the obtainment of blood samples. Those general difficulties are even higher, when samples shall be collected from wild animals.

Due to financial and ethical restrictions, possibilities to capture wild animals are very rare. Field trips are expensive and quotas are highly restricted. A large number of individuals are generally involved in the planning and execution of field trips and since as much data as possible shall be obtained from one trip, organizational efforts are immense. For this study, sampling was carried out by researchers from the Norwegian and Spanish collaborating institutes.

Restrictive and often unexpected conditions in the wild pose a scenario very different from what one is used to working with the sampling of domestic animals. Considering, that boats need to travel for longer periods of time to and back from the site of sampling and that the sampling itself takes several days, the maintenance of samples in perfect conditions is also difficult. Liquid nitrogen tanks were available in some but not all of the sampling events, and samples were often under suboptimal conditions and exposed to repeated freezing and thawing events before actual testing. For example, swab samples from the West Ice trip in

2010 were kept at -20°C and not -80°C for further use, adding another variable that may account for the fact that all samples tested negative in qPCR later on.

To justify the disruption on animals imposed and on their habitat and the high financial efforts, samples must be used for as many tests as possible and a multitude of opinions and requests are coming together and need to be considered. The final outcome and decisions may not in all cases fit every individual's needs, however. In the discussion of results obtained, it will become clear that missing information and incomplete sample sets often made interpretations and conclusions difficult. Thorough planning of field studies therefore is one of the key aspects in studies, particularly when working with samples so difficult and expensive to obtain.

1.3. Inhomogeneity of sample subsets generally and statistical analyses

IV.1.2. already described general problems in obtaining samples from wild seals. Samples obtained under those difficult conditions pose further problems, however. The number of animals that can be tested is, due to the conditions described and for ethical and animal welfare reasons, generally low and if several species are involved, numbers for every individual subset often end up being even lower. Only descriptive statistics could be applied, since total numbers were small and conditions were very invariable. Since very little data has been published so far, descriptive statistics were advised and evaluated to be appropriate by experts (personal communication Institute for Biometry, Freie Universität Berlin).

Contrary to investigations on domestic species, even key parameters, such as gender, age or health status, are difficult to obtain in seals. For aging, a tooth needs to be extracted from each individual and analyzed, a process that can take up to years for large sample subsets. In few cases, where age determination was missing for individuals, the age was estimated based on the BMI of animals. According to BMIs of harbor seals captured in Svalbard, animals with a BMI of 30 and lower were always pups and animals with BMIs ranging up to 30 were therefore considered pups.

For samples from other expeditions such as the testing of Antarctic seals, it was only distinguished between pups and adults (Norwegian expedition) or no age data was available at all (Spanish expedition), and analyses of data in that regard were therefore not possible. Especially including retrospective data for which study design was done more than ten years ago and planned to suit different experimental needs, one needs to often deal with limited information and an increased interpretation range.

1.4. Problems with the extraction of DNA from swabs

Swab samples of different groups from different expeditions in various years were not only collected in different sample buffers but later proceeding was also not carried out homogeneously.

During the beginning of the studies, samples were extracted with the single column DNeasy blood and tissue kit from Qiagen (Hilden, Germany). To increase efficiency and deal more quickly with large numbers of samples, it was decided to use the 96-well formate of Qiagen's DNeasy blood and tissue kit. Unfortunately, centrifugation of samples in this format could not be achieved and extractions with the 96-well kit failed. According to positive experiences with the DNA/RNA Virus Mini kit (Stratec, former Invitek, Berlin, Germany) for diagnostic purposes, the DNeasy blood and tissue kit (Qiagen) was replaced by the single column extraction DNA/RNA Virus Mini Kit (Stratec) and successfully used for the rest of samples.

DNA from samples obtained during the expedition to Antarctica by the Spanish group of researchers was extracted in Spain using Qiagen's DNeasy blood and tissue kit.

Nasal swabs from harbor seals captured on Svalbard 2009 were kept in RNAlater with the initial idea of morbillivirus testing later. Only tissue samples and no single cells (and hence no blood/liquid or swab samples) can, unfortunately, be preserved in RNAlater and extraction with general methods completely failed on those samples. Unknown components of RNAlater did not allow the binding powder to dissolve and precipitations clogged the columns.

2. Discussion of data obtained

2.1. Phylogenetic studies

Limited phylogenetic analyses were approached in the beginning, particularly as a basis for the establishment of the qPCR assay. Partial sequences of gB and Pol were compared to published sequence information for primer and probe design and compared to sequence information on related viruses of terrestrial mammals such as cats, dogs and horses.

Glycoproteins are located in the envelope of herpesviruses and serve for receptor binding and specific antibody recognition (Tunback et al., 2000). They are, therefore, under constant selection pressure, causing genetic and antigenetic variability.

Both, Pol and gB are located within a gene block that is conserved throughout all herpesviruses (Kouzarides et al., 1987). The herpesviral DNA polymerase gene is highly conserved and often used for diagnostic purposes. Particularly the low selection pressure of

an essential gene is thought to account for the high degree of conservation (Miranda et al., 2005).

Interestingly, sequence alignments and comparisons of gB sequences in our study revealed, that gB sequences are more conserved than viral polymerase sequences, however. For this reason, gB was chosen as a target for the qPCR assay.

Comparison of sequences of seals living in Arctic / Atlantic / European waters and the Pacific could show some degree of variation, most of which were non-synonymous mutations. How long these seal populations have been isolated from each other and whether there is a possibility of pathogen transmission through migration or vectors remains to be elucidated. Partial gB sequences from qPCR positive harbor seals all revealed one single, non-synonymous, nucleotide change compared to other European sequences, possibly suggesting that the world's northernmost harbor seal population is living isolated from other harbor seal populations living further south.

2.1. Harbor seals Svalbard

Little is known about PhHV-1 in wild harbor seals. 124 wild harbor seals off the coast of Russia and Alaska were tested for PhHV-1 seroprevalence during 1978 – 1994, revealing 77% positive animals (Zarnke et al., 1997). To our knowledge, swab samples from wild seal populations have so far never been tested for PhHV-1 DNA and this, hence, is the first report on the detection of herpesviral DNA in ocular swabs from 2009 and ocular and nasal swabs from 2010. Furthermore, the harbor seal population off Svalbard was repeatedly tested serologically from 1998 to 2000 and again in 2009 and 2010.

All animals tested positive for PhHV-1 DNA in swabs were one year and younger. In 2009, five animals were pups and one was one year old; both animals tested positive in 2010 were one year old. The hypothesis, that phocine herpesvirus DNA could be found in adult animals during reactivation was not undermined and most likely only animals with primary infection were detected by qPCR. Assuming that all animals positive for PhHV-1 DNA were diagnosed during primary infection, when high viral loads should be present in both nasal and ocular swabs leads to the conclusion that DNA yields from swab samples were generally low. For 2009, only ocular swabs were available and copy numbers ranged between very low (< 3 copies) and very high (almost 4000 copies). In samples obtained in 2010, DNA from ocular and corresponding nasal swabs was extracted and tested. Since low copy numbers were detected in samples from animals with primary infection already, PhHV-1 DNA in animals during low level viral shedding is expected to be below the detection limit. Hence, the

hypothesis that PhHV-1 has a reactivation site in eyes of seals could can neither be rejected nor confirmed.

Interestingly, none of the animals sampled were reported to have obvious health problems or signs of disease, although one would expect animals with primary infection to show general signs of malaise and symptoms of upper respiratory disease.

Serum samples from most animals were tested for anti-PhHV-1 antibodies. Samples from 2009 were tested by SNT and ELISA and statistical analyses showed a positive correlation. Therefore, the ELISA could be used for further testing. Furthermore, serum samples from previous expeditions in 1998, 1999 and 2000 were available for testing. This enabled retrospective monitoring of the population over a 12-year period. 92%, 88%, 100%, 72% and 87% (years 1998 – 2000 and 2009 / 2010 respectively) of animals tested positive and are in agreement with overall high seroprevalences reported previously (Zarnke et al., 1997).

Serological data was only available for six out of eight animals that were tested positive by qPCR. For all three animals less than one year of age, serum was available. Two tested positive and one was found without detectable anti-PhHV-1 antibodies. The latter was most likely captured in the early stage of primary infection, whereas the other two animals may have been in a later phase of disease at the time of sampling or already carried antibodies from an infection as neonates. Animals testing positive for DNA but negative for antibodies were detected with high copy numbers of viral DNA. Reactivation events in seals one year of age cannot be completely ruled out. The fact that no animals from other age groups were detected with PhHV-1 DNA does not support the theory that DNA could be found during reactivation, however. Out of four animals under one year of age, positive by qPCR and with serum available for testing, two had no antibodies and one had a high titer of 1:12800. Antibodies detected in the newborn seal could either derive from the primary infection in case the pup was tested during later stages of infection or could be maternally derived antibodies.

For 2009 and 2010, data was available on the moulting status at the time of sampling. Change of coat is a stressful time for the animals in which they reduce or stop food intake. Additionally, moulting takes place after parturition and lactation and therefore is the final phase of a straining and catabolic phase for female animals. But stress levels are elevated during courting and mating in male animals, often associated with territorial fights. Assuming that reactivation is, as seen from herpesviruses in other animal species and in humans, triggered by socially and physically stressful events, and assuming that reactivation events are going along with a rise in antibody titers through the repeated representation of the pathogen to memory cells, the question arose, whether moult presented such a stressful event for seals. Could animals in moult have elevated antibody titers? Comparing animals in moult with others, the first population showed higher seroprevalences. Due to low sample numbers, the difference could not be confirmed to be statistically significant.

No gender specific differences were determined regarding PhHV-1 seroprevalence. In the comparison of different age groups (exemplarily shown for 1998). Seroprevalences were high in neonates and animals two years of age and older. Titers were rising with age. High seroprevalences in pups are likely caused by maternal antibodies. So far, herpesviral disease through primary infection has been reported to occur in pups (Martina et al., 2002). Rising of titers in older animals could be explained by reactivation events with corresponding immune response (Goldstein et al., 2003). So far, the site of reactivation and virus detection during reactivation events in adults could not be determined and achieved, however.

Annual changes of seroprevalences within populations could be related to higher numbers of primary infections and / or higher numbers of animals undergoing reactivation. Herpesvirus reactivation events can be influenced and triggered through a multitude of factors, disturbing the fine balance between virus and host during latency (Croen, 1991; Field et al., 2006). For herpesviruses of domestic animals factors such as crowding or other stressful events in their habitat are known to have the potential to disequilibrate the latent virus - host cohabitate (Gaskell and Povey, 1982; Tanaka and Mannen, 2003; Thiry et al., 1987). Factors influencing ocular disease in seals such as increased UV radiation, pH imbalances and opportunistic pathogens (Dierauf, 2001) are also considered factors triggering reactivation. Assumptions about PhHV-1 and ocular disease have only been made for seals in captivity, so far, and – to our knowledge – challenges have not been conducted. Factors that may initiate and influence herpesvirus reactivation in free-ranging animals are even more numerous and harder to evaluate and to detect than those in captivity. Changes in habitat, climatic conditions, population density or health status and other infectious diseases are only few possibilities that may, alone or in combination, provoke differences in prevalence of primary infection and reactivation and hence overall seroprevalences in different years. It cannot be omitted that populations tested over the years were very inhomogeneous. For example high variances in the fraction of pups (0% - 38%) were recorded. As it was shown that seroprevalences are influenced by the age of animals, no final conclusions can be made regarding overall seroprevalences unless completely comparable / identical populations are tested each year. Since part of the data was used retrospectively, study design had no influence on the sampling. Generally, working with wild populations of seals located in one of the world's least hospitable environments, the availability of such homogenous sample subsets is highly unlikely.

Virus isolation was attempted but, possibly due to partly sub-optimal maintenance conditions for swabs and tissues, no virus could be isolated. For a number of passages, CPEs could be detected but effects were either artifacts caused by toxic effects or, the causative agent failed to be further propagated in cell culture. No antibody was at hand at the time so that anti-

PhHV-1 immunofluorescence assays could not be made and CPEs were lost before sufficient amounts of infected cells could be grown for electron microscopy.

2.2. Harp seals and hooded seals West Ice 2009, 2010

It has been reported, that harp seals and hooded seals in the West Ice and in the East Ice were tested for PhHV-1 seroprevalences in 1991 / 1992. Harp seals had seroprevalences of 29 – 41% and hooded seals were positive with prevalences of 4 – 7% (Stuen et al., 1994). Ocular and nasal swabs collected in 2009 and 2010 in the West Ice from harp seals and hooded seals as well as larger numbers of ocular, nasal and genital swabs and tissues of different organs were available for testing from the expedition in 2009. Vertical transmission was addressed in Pacific seals before (Goldstein et al., 2004) but to our knowledge this is the first report on PhHV-1 DNA detection in genital swabs of free-ranging animals. Overall, numbers of positive swab samples were higher than in harbor seals tested off Svalbard. Despite high seroprevalences in harbor seals off Svalbard, the few samples from the West Ice tested negative in SNT or showed very low titers, which remains ambiguous. Unfortunately, no age data is available for harp seals from the West Ice up to the present. All hooded seals were pups, however. Information on age and seroprevalence of individuals found with PhHV-1 DNA in swabs would be needed for interpretations regarding reactivation and possible vertical transmission. The finding of virus DNA in genital swabs raises the question whether PhHV-1 may, as is the case in herpesviruses of domestic animals such as BHV-1 or EHV-1 (Burrows and Goodridge, 1975; Smith, 1997; van Nieuwstadt and Verhoeff, 1983), be involved in abortion events, a hypothesis that will have to be investigated further. Higher DNA copy numbers (> 1000) were mainly found in ocular and nasal swabs whereas copy numbers in genital swabs were rather low. Although comparable large numbers of animals were tested, hardly any complete sets of swabs (ocular, nasal and genital) were available for testing due to problems in DNA extraction described in IV.1.4.. Interestingly, in none of the animals with PhHV-1 positive ocular swabs, DNA could be detected in nasal swabs and vice versa. Only in two animals, matching ocular and genital swabs were positive. Nasal swabs were not available for both animals. Copy numbers were not exceptionally high and in both cases lower in genital swabs. No significant differences could be seen between the two different species.

In none of the DNA samples extracted from tissues (liver, lung, kidney, spleen and for some animals reproductive organs) viral DNA could be detected with the developed assay. Since DNA could be detected in seals with generalized PhHV-1 infection by conventional PCR, generally associated with lower sensitivity (Goldstein et al., 2005), it is assumed that animals

found with qPCR positive swab samples were not at the stage of generalized infection at the moment of testing. None of the animals was reported to show clinical signs of disease. This in turn leads to the assumption that DNA in swab samples was found in seals during reactivation events. Since all hooded seal samples were from pups and no age data is available for harp seals, it cannot be excluded that the animals tested positive were captured during primary infection as seen for harbor seals off Svalbard.

A smaller number of swab samples (only nasal and ocular) was available for harp seals and hooded seals sampled in 2010, none tested positive for PhHV-1 DNA by qPCR. A number of variables could potentially account for false negative results. To name few of them, factors such as different swabbing techniques in the field, different storage of samples, the transport of swabs to Berlin and extraction not on-site in Norway as done for all other samples may have had an impact. Storage of samples at -20°C and not -80°C before transport to Berlin under variable conditions (not frozen) over a long period of time are considered problematic. Enveloped viruses were shown to lose infectivity even when stored at 90°C, being highly labile at -20°C with better stability at 4°C (Melnick, 1965; Wallis and Melnick, 1968). Despite the original recommendation, that the stability of frozen swab specimens was limited to only six weeks (Farkas et al., 1996), the evaluation of quantitative stability of clinical herpesvirus specimen revealed no decrease in detectability via qPCR over time, even when stored at -20°C (Jerome et al., 2002).

As for harbor seals samples from Svalbard, virus isolation was attempted and failed, for which, again, most likely account the loss of infectivity under suboptimal storage conditions.

2.3. PhHV-1 in the Arctic

Swab samples from the Arctic were obtained from three different species, hooded seals, harp seals and harbor seals at different locations under varying conditions. Overall comparisons therefore need to be interpreted with care. Considering all the variables and difficulties of the study, our qPCR data indicates that harp seals and hooded seals show higher numbers of animals with PhHV-1 infection or reactivation than harbor seals. All hooded seals and harbor seals were known to be young animals up to one year of age and need to be considered as primarily infected. Our data lead to the conclusion that PhHV-1 is common in Arctic seals, a fact that was also supported by high seroprevalences of harbor seals over time. It cannot be concluded whether the differences in species or in location account for the variation in PhHV-1 prevalence.

2.4. Antarctic fur seals, Weddell seals, Ross seals and crabeater seals Antarctica 2000-2002

Only two reports have been published on PhHV-1 seroprevalences in Antarctica before. For both studies, the same subset of samples from an expedition (German research vessel Polarstern) was investigated by SNT (Harder, 1991; Stenvers et al., 1992). Different results were obtained: Weddell seals showed high seroprevalences (100% and 72% respectively) and were also seen to suffer from respiratory disease at the time of sampling. Results for crabeater seals differed widely: whilst Harder et al. found all three animals to be positive for PhHV-1 antibodies, Stenvers et al. reported the two samples, which were tested, as negative.

All Weddell seals in this study, captured between 2000 and 2002 (n = 20), tested positive in ELISA with titers between 1:3200 and 1:12800. Only a small number of crabeater seals (n = 9) was captured and four animals had similar antibody levels with titers ranging from 1:6400 to 1:12800.

Ross seals have, to our knowledge, not been tested for PhHV-1 seroprevalences before and compared to other species tested, fewer numbers of animals (3 out of 20) were found with anti-PhHV-1 antibodies. Since Ross seals, Weddell seals and crabeater seals were sampled at the same location, differences in seroprevalence appear to be species specific and not dependent on the capture site.

PhHV-1 seroprevalences in Antarctic fur seals have not been addressed before either. Since Antarctic fur seals are not a phocid but an ontarid species, it was questionable whether cross-reactivity would allow the detection of seal herpesvirus seroprevalences in the species. A total of 58% of animals tested positive in the PhHV-1 ELISA, however, indicating that cross-reactivity was sufficient. Exact age data for all animals were not available but it was distinguished between pups and adult seals. Interestingly, in contrast to observations in harbor seals, seroprevalence and titers were higher in pups than in adults. Again, large confidence intervals originated from the small number of pups within the population (13 out of 95), only a trend could be seen and the difference was not significant. The observed trend is, however, contrary to findings in harbor seals. In Antarctic fur seals, PhHV-1 may, therefore, be even more of a problem for young animals. One explanation may be that reactivation events occur less frequently in adult Antarctic fur seals, leading a decrease in titer over time and with age.

2.5. Antarctic fur seals, Weddell seals, Ross seals and Southern elephant seals Antarctica 2010

During an expedition 10 years later, serum samples from Weddell seals, Antarctic fur seals and, for the first time, Southern elephant fur seals were obtained and tested. The number of Antarctic fur seals tested positive for anti-PhHV-1 antibodies was relatively low (14%). Unlike previous reports and data obtained in 2000 – 2002. Numbers of positive Weddell seals were lower as well. For the first time, serum of Southern elephant seals was tested and four out of five animals had high anti-PhHV-1 antibody titers. Although sampling locations were in relative close proximity to each other, one sample obtained from an animal off a different island was negative. The small sample number does not allow final conclusions but PhHV-1 seroprevalences may be dependent on the location of animals. Looking at the three different species sampled, two (Antarctic fur seals and Southern elephant seals) showed less or no seroprevalence on Deception Island, whereas seroprevalences in Weddell seals were slightly lower on Avian Island. Again, small population numbers only allow the description of trends and the impact of location and species would have to be investigated further with equivalent sample numbers from the different islands. No age data was available for the animals tested. It was seen before that seropositivity was lower in adult Antarctic fur seals, which may partly account for low seropositivities in Antarctic fur seals if adult animals were tested only.

Nasal swabs were taken from all animals but no PhHV-1 DNA could be detected by qPCR. Again, as described before, various factors may have led to false negative results but no conclusions can be drawn. No PhHV-1 has been isolated from or detected in Antarctica to date and therefore no sequence data is available. As shown in III.3., phocid herpesviruses circulating in animals from Arctic / Atlantic and Pacific waters differ genetically but occasional genetic exchange is not impossible. Transmission between Arctic and Antarctic seal species can be excluded, however. It can be expected that sequences of phocine herpesviruses in Antarctic species are too diverse from the sequences the qPCR assay was based on, accounting for a failure in detection with the highly specific assay. Furthermore, Antarctic fur seals are ontarids and despite the cross-reactivity with the PhHV-1 ELISA, PhHV-1 is a virus of phocids and ontarids have their own herpesvirus species (Maness et al., 2011).

2.6. PhHV-1 seroprevalence in Antarctica

Seroprevalences were tested at two different parts of Antarctica with a gap of ten years between sampling. Both studies address seroprevalences in Antarctic fur seals for the first

time and, interestingly, results differed largely (58% and 14%). Location as well as time point of testing may, besides other non-determined variable factors, account for the differences. Susceptibility of Weddell seals, as reported previously (Harder, 1991; Stenvers et al., 1992), was confirmed and our data supports the report of Harder et al. that crabeater seals are infected with PhHV-1. Additionally we were able to test two species that had not been tested for their susceptibility to PhHV-1 before: Ross seals and Southern Elephant seals. Low sample numbers make an interpretation difficult but, overall, it could be shown that both species are susceptible to the virus.

2.7. PhHV-1 in different environments

Serological data obtained from Arctic locations mainly confirmed previous reports for PhHV-1 in different seal species and analysis brought up new questions regarding for example the impact of molting and of reactivation on antibody titers. The introduction of a sensitive qPCR assay for the detection of DNA in swab and tissue samples, as well as serological data confirmed the infection of Arctic species with PhHV-1. Negative qPCR data from Antarctic phocid species is likely constituted by the genetic diversity of PhHV-1 isolates in the Northern and Southern hemisphere. Hardly any serological data regarding PHV-1 had been collected in Antarctic seal species before and our studies could even show the susceptibility of two additional species, Ross seals and Southern elephant seals.
The variability in sample numbers and data available for each of the subsets makes overall comparisons difficult. It can be concluded, however, that PhHV-1 occurs in seals worldwide with species-specific and site-specific differences.

3. Conclusions and outlook

The study presents a new and worldwide overview on the occurrence of PhHV-1 in free ranging seals worldwide, for one of the locations over a long time period. A qPCR assay was developed but despite the high sensitivity, only low copy numbers of virus DNA could be detected in ocular and nasal swabs from harbor seals one year and younger, most likely captured and sampled during primary infection. Interestingly, in hooded seals and harp seals, PhHV-1 DNA could also be detected in genital swabs. The assay is therefore a suitable method for the detection of PhHV-1 infection but difficulties in obtaining, maintaining and proceeding of swab samples have to be considered and re-evaluated for further use. Since no virus DNA could be detected in animals captured in Antarctica, virus isolation and

sequencing from Antarctic samples is desired so that questions regarding specificity of the PCR protocols could be addressed.

The PhHV-1 ELISA used for the evaluation of seroprevalences was shown to correlate with results obtained in SNT and, therefore, is considered a suitable method for the detection of PhHV-1 in populations of free ranging seals. The assay showed cross-reactivity with seal herpesvirus in Antarctic phocids and even ontarids and can be used for testing of seals worldwide. Serological data showed high seroprevalences in harbor seals and some Antarctic species and anti-PhHV-1 antibodies could be detected for the first time in Ross seals and Southern elephant seals, indicating susceptibility to seal herpesvirus of the respective species.

Further investigation of captive and potentially free ranging seals with symptoms of ocular disease will be needed to answer the question whether PhHV-1 is involved in the etiology of ocular anterior segment disease of seals. Repeated testing of swab samples from diseased animals over a time course involving the treatment with corticosteroids (immunosupression, trigger), monitoring the severity of disease, would further be of interest.

The question whether PhHV-1 is involved in abortion events in seals is even more difficult to address. Breeding is rare in captivity and the number of abortions is low. Some zoos and rehabilitation facilities may be able to closely monitor animals breeding in captivity for PhHV-1 serologically and via (quantitative) PCR however, so that more information could be gathered in the end. Testing of more genital swabs from free ranging seals with corresponding age data would be valuable, as well.

Finally, the isolation and sequencing of more virus isolates from different species and different locations worldwide is desired for a better understanding of the distribution of seal herpesviruses and one day, the question may be answered whether seal herpesvirus has evolved in marine mammals for a long time or whether it was, comparable to PDV, introduced by terrestrial carnivore species.

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Appendix

Table A-1: List of materials

Material	Manufacturer
Cell culture flasks	TPP
Cell culture plates (6-, 12-, 24-, 96-well)	TPP
Cryo tubes	Hartenstein
Hi Yield Gel/PCR DNA Fragments Extraction Kit	SLG
Gloves, gloves nitril	Flexam, Roth
Maxisorp ELISA 96-well plates	Nunc
Petri dishes	Hartenstein
Pipette tips	VWR
Pipettes	TPP
Polypropylene tubes	TPP / Hartenstein
qPCR 96-well plates	ABI
Reaction tubes (0.2 ml, 1.5 ml, 2 ml)	Hartenstein
TOPO TA Cloning Kit	Invitrogen

Table A-2: List of equipment

Equipment	Model/type	Manufacturer
Bunsen burner	Typ1020	Usbeck
Cellculture incubator	Excella ECO-170	New Brunswick Scientific
Cell counting chamber	Neubauer improved	Assistent
Centrifuges	Centrifuge 5424 Centrifuge 5804R Galaxy Mini	Eppendorf Eppendorf VWR

Equipment	Model/type	Manufacturer
Cryo storage system	Cryo1°C Freezing container	NALGENE
ELISA reader	TriStar LB 941	Berthold Technologies
Freezer	-20°C -80°C	Liebherr GFL
Gel electrophoresis chambers	Mini Elektrophorese System SUB-Cell GT	VWR Bio-Rad
Ice flaker	AF100	Scotsman
Laminar flow		Bleymehl
Microscope	Axiovert S100 AE 20	Zeiss Motic
Microwave		Bosch
Nanoscale Spectrophotometer	NanoDrop2000	ThermoScientific
Nitrogen tank	ARPEGE70	Air liquide
PCR cycler	T-Gradient GeneAmp PCR System 2400	Biometra Perkin Elmer
pH-meter	HI 223 Calibrations Check	Hanna Instruments
Pipettor	Accu-jet pro	BRAND
Power supply	Power Source 250V	VWR
qPCR cycler	7500 Fast	ABI
Refrigerator	KS 2460	EBD
Shaking incubator for bacteria	Incubator Shaker innova 44	New Brunswick Scientific
Spectrophotometer	BioPhotometer	ThermoScientific
Thermomixer	Thermomixer 5326 Thermomixer comfort Thermomixer compact	Eppendorf Eppendorf Eppendorf
Ultracentrifuge	L8-70M	BECKMAN
UV detection system / Gel documentation	Transiluminator Bio-Vision-3026 Printer P93D	PeqLab Mitsubishi

Equipment	Model/type	Manufacturer
Vacuum aspirator	Vacumat130	H.Saur
Vortexer	VTY-3000L	Hartenstein
Waterbath	TW8 Water Bath Shaker C76	Jubalo New Brunswick Scientific
Scales	ALC-2100.2 ALC-1100.2	Acculab Acculab

Table A-3: List of reagents

Reagent	Manufacturer
Acetic acid, glacial	AppliChem
30 % Acrylamide	Roth
Agarose	Roth
APS (ammonium persulfate)	Merck
Bacto-Tryptone	BD
Bacto-Yeast extract	BD
BSA (bovine serum albumine)	Biomol
$CaCl_2$ x $2H_2O$	Roth
Carboxymethylcellulose	Sigma-Aldrich
CH_3CO_2K	Merck
Crystal violet	Merck
DMSO	Merck
Dithiothreitit (DTT)	Merck
ECL Plus Detection System	GE HealthCare
EDTA	Merck
Ethidiumbromid	Roth
Ethanol 95 % for analysis	Merck
Fastmix lowROX (qPCR master mix)	VWR

Reagent	Manufacturer
FBS	Biochrom AG
Glycerin	Merck
HCl	Roth
H_2SO_4	Merck
KH_2PO_4	AppliChem
Isopropanol	Merck
KCl	Merck
MEM	Biochrom AG
Methanol	AppliChem
$MgCl_2$	Merck
$MgSO_4$	Merck
$MnCl_2$ x $4H_2O$	Merck
Na_2EDTA x $2H_2O$	Serva
NaCl	Merck
Na_2CO_3	Merck
$NaHCO_3$	Merck
NaOH	Merck
Phenol/Chloroform	AppliChem
Pipes	Roth
Protein A (peroxidase-conjugated)	US Biological
Restriction enzymes	NEB
RNaseA	AppliChem
SDS	Serva
Secundary antibody anti-mouse (rabbit)	Rockland
Taq-Polymerase	Genescript, Peqlab, NEB
TEMED (Tetramethylethylendiamin)	Sigma
TMB (Tetramethylbenzidine)	Sigma

Reagent	Manufacturer
Tris-base	AppliChem
Trypsin	GIBCO
Tween-20	Merck

Table A-4: List of solutions and composition

Solutions	Composition
1000x Ampicillin	100mg/ml in ddH_2O
Crystal violet	0.5 % Crystal Violet 25 % Methanol
LB	1% Bacto-Tryptone 0.5% Bacto-Yeast extract 1 % NaCl
SDS Page Loading Buffer	4% SDS 0.1M DTT 20% glycerol 0.004% bromophenol blue 0.125M Tris HCl
P1 for alkaline lysis protocol	50mM Tris 10mM EDTA, pH 8.0
P2 for alkaline lysis protocol	0.2N NaOH 1% SDS
P3 for alkaline lysis protocol	3M CH_3CO_2K, pH 5.5
PBS	2mM KH_2PO_4 10mM Na_2HPO_4 137mM NaCl 2.7 mM KCl, pH 7.3

Solutions	Composition
PBST	2mM KH_2PO_4 10mM Na_2HPO_4 137mM NaCl 2.7 mM KCl, pH 7.3 0.05 % Tween-20
100x Penicillin/Streptomycin	100U/ml and 0.1 mg/ml in MEM
SDS Page 5 % stacking gel	0.125M Tris-base pH 6.8 0.1% SDS 0.5% TEMED 0.1% APS 5% Acrylamide
SDS Page 10% running gel	0.375M Tris-base pH 8.8 0.1% SDS 0.4% TEMED 0.1% APS 10% Acrylamide
SOB	2% w/v bacto-tryptone 0.5% w/v bacto-yeast extract 10mM NaCl 2.5mM KCl pH 7-7.5
TAE	50mM Tris-base 2.5mM $Na_2EDTA \times 2H_2O$ 25mM Acetic acid 99% pH 8
TB	10mM Pipes 15mM $CaCl_2 \times 2H_2O$ 250mM KCl 55mM $MnCl_2$ pH 6.7
TE	10mM Tris-base 1mM $Na_2EDTA \times 2H_2O$, pH 7.5

Table A-5: List of Software

Software	Provider
ClustalX	www.clustal.org
FIGTree	www.tree.bio.ed.ac.uk/software/figtree/
GraphPad QuickCalcs	www.graphpad.de
Office	Microsoft
PHYLIP	www.evolution.genetics.washington.edu
PrimerExpress3.0	ABI
PrismX	www.graphpad.de
VectorNTI	Invitrogen

List of publications

Roth, S.J., Hoper, D., Beer, M., Feineis, S., Tischer, B.K., Osterrieder, N., 2011, Recovery of infectious virus from full-length cowpox virus (CPXV) DNA cloned as a bacterial artificial chromosome (BAC). Vet Res 42, 3., [open access online] http://www.veterinaryresearch.org/content/42/1/3

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das Neves, C.G., **Roth, S.**, Rimstad, E., Thiry, E., Tryland, M., 2010, Cervid herpesvirus 2 infection in reindeer: a review. Vet Microbiol 143, 70-80.

Talks and poster presentations (conference proceedings)

Roth, S.J., Osterrieder, N., Feineis, S., Tischer, B.K., 2009, talk
The cowpox virus BAC – a tool for the determination of immunomodulatory factors. Workshop der GfV AG Immunobiologie, Deidesheim

Roth, S.J., Osterrieder, N., Tischer, B.K., 2009, Poster
Ocular manifestations of phocid herpesvirus type 1 in pinnipeds. 3rd ESVV Veterinary Herpesvirus Symposium, Greifswald – Insel Riems

Roth, S.J., Osterrieder, N., Feineis, S., Tischer, B.K., 2010, poster
Generation of a cowpox virus (CPXV) bacterial artificial chromosome. 4th ESV European Congress of Virology, Lake Como, Italy

Roth, S.J., Tischer, B.K., Feineis, S., Osterrieder, N., 2010, poster
Generation of an infectious BAC clone of cowpox virus. XVIII International Poxvirus Symposium, Sedona, AZ

Roth, S.J., Harbison, C.E., Atkins, H.M., Hafenstein, S.I., Stucker, K.M., Parrish, C.R., 2011, poster
Development of contraceptive vaccines based on canine parvovirus virus like particles. 30th Meeting of the American Society for Virology, Minneapolis, MN

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 4. November 2011 Swaantje Juliane Roth