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The transmission of phocine herpesvirus-1 in rehabilitating and free-ranging Pacific harbor seals (*Phoca vitulina*) in California

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Abstract

Phocine herpesvirus-1 (PhHV-1) causes regular outbreaks of disease in neonatal harbor seals (*Phoca vitulina*) at rehabilitation centers in Europe and in the U.S. To investigate transmission of this virus samples were collected from harbor seal pups during exposure studies at a Californian rehabilitation center from 1999 to 2002 and from free-ranging harbor seals off central California during the same period. The exposure studies provided evidence that PhHV-1 can be transmitted horizontally between animals most likely through direct contact with oro-nasal secretions. However vertical transmission may also occur, as adult female harbor seals were found to be shedding the virus in vaginal and nasal secretions, and premature newborn pups had evidence of early infection. Results also indicated that PhHV-1 infections were common in both free-ranging (40%, 49/121) and rehabilitating (54%, 46/85) young harbor seals, during the spring and early summer. This timing, which correlated with pupping and weaning, suggested that the majority of animals were infected and infective with PhHV-1 between pupping and breeding.

Keywords: Harbor seal; *Phoca vitulina*; Phocine herpesvirus; Viral transmission; PCR; ELISA

1. Introduction

Phocine herpesvirus-1 (PhHV-1) is an alpha herpesvirus that has been isolated from harbor seals (*Phoca vitulina*) in both the eastern Atlantic and Pacific oceans (Osterhaus et al., 1985; Gulland et al.,

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1997). This virus is known to cause regular outbreaks of disease, often resulting in large numbers of deaths of neonatal harbor seals undergoing rehabilitation, causing up to 23% mortality in some years (Harder et al., 1997; King et al., 2001; Martina et al., 2002).

Studies of PhHV-1 infections to date have utilized techniques such as virus isolation, polymerase chain reaction (PCR) or enzyme linked immunosorbent assays (ELISA) to investigate outbreaks of disease (Harder et al., 1997; King et al., 2001; Martina et al., 2002). Both Harder et al. (1997) and Martina et al. (2002) focused on outbreaks occurring in Europe and showed that the majority of young rehabilitating harbor seals exposed to the Atlantic PhHV-1 isolate developed overt clinical signs of upper respiratory disease, and the severity of disease was inversely correlated with age (fatal infections occurring in newborns but not in older seals). A study of infection in juvenile seals (4–5 months old) exposed to the European isolate of PhHV-1 via inoculation and direct contact supported this observation, as these older animals exhibited mild signs of illness and subsequently recovered (Horvat et al., 1989). In contrast, clinical signs associated with PhHV-1 infections in harbor seals in the Pacific include seizuring and sudden death as a result of hypoglycemia, generalized depression and failure to thermoregulate. Necropsy findings in Pacific harbor seals have primarily associated PhHV-1 with adrenal and sometimes liver lesions in newborn harbor seals dying in rehabilitation in central California (Gulland et al., 1997). An understanding of disease transmission in Pacific harbor seals would assist in designing prevention and animal management protocols with respect to this disease in rehabilitation centers. Although features of PhHV-1 infection have been studied in Atlantic harbor seals, the mode of transmission has not specifically been addressed, and since the pathogenesis of this infection in the Pacific appears to differ from that in the Atlantic, further transmission studies were needed in California.

Little is known about the prevalence of PhHV-1 infections in adult and subadult harbor seals or the maintenance of the pathogen in free-ranging populations. Intranuclear inclusion bodies typical of a herpesvirus have been noted in tissues collected from a limited number of harbor seals dying on beaches along the eastern Pacific, suggesting that herpesvirus infections probably occur in these animals (Spraker et al., 1994; Gulland et al., 1997). Results from a recent serosurvey for PhHV-1 in harbor seals around North America showed that exposure appears to be common in free-ranging seals and that PhHV-1 specific antibodies increase with age, with almost all adults testing seropositive (Goldstein et al., 2003). If PhHV-1 infection persists in seals, as occurs with herpesviruses in most mammalian hosts (Honess and Watson, 1977), adult seals may provide a source of virus to naïve individuals. Transmission could occur during periods of viral reactivation either vertically from mother to pup or horizontally between pups through direct contact or by a combination of these routes, allowing a cycle of infection to persist from year to year.

The first objective of this study was to investigate the mode of PhHV-1 transmission in young pups and document the temporal sequence of events following exposure. Specifically, we wanted to determine whether horizontal transmission occurred between animals through direct contact and whether there was evidence for vertical transmission from mother to newborn. For this objective we serially sampled Pacific harbor seal pups housed at a rehabilitation center on the central California coast during the rehabilitation seasons from 1999 to 2001. The second objective was to characterize the distribution of PhHV-1 infection in a free-ranging harbor seal population along the central California coast in order to investigate whether PhHV-1 infections were occurring in free-ranging seals or were unique to animals undergoing rehabilitation.

2. Materials and methods

2.1. Animals and samples

All harbor seals (free-ranging $n = 270$, rehabilitating $n = 85$) sampled for this study were captured or stranded on the California coast between January 1999 and March 2002. Eighty-five seals [62 pre-weaned pups (up to 2 months old), 18 recently weaned pups (2–3 months old), three subadult males (between 2 and 5 years of age) and two adult females (greater than 4 years of age)] that stranded live on the California coast (37°42′ to 35°59′N, 123°05′ to 121°30′W) from January 1999 to December 2001 were transported to

The Marine Mammal Center, Sausalito, CA, USA for treatment. Samples were collected from 28 seals that were admitted during 1999, 37 in 2000 and 20 in 2001.

Upon admission, the age and sex of each seal were determined based on external morphological characteristics (Bigg, 1969a), and all animals were weighed, measured, tagged, examined clinically and sampled. Blood was drawn from the extradural intravertebral vein or the interdigital vein in the rear flipper as described by Bossart et al. (2001) and placed into vacutainers containing either ethylenediaminetetra-acetic acid (EDTA) or serum separation gel (Vacutainer, Becton Dickenson, Rutherford, NJ, USA). Nasal, oral, rectal, ocular and vaginal (females over 1 year of age) swabs were collected using sterile cotton tipped applicators, placed in sterile vials and frozen for DNA extraction.

Mononuclear leukocytes were isolated from whole blood samples for DNA extraction by placing the buffy coat layer over a ficoll mixture (Isolymph, density 1.077, Gallard-Schlesinger Industries Inc., Carle Place, NY, USA) and centrifuging at 400 × *g* for 30 min. Genomic DNA was extracted from mononuclear leukocytes and swab samples using a silica-gel-membrane based commercial kit (DNeasy Tissue Kit®, Qiagen Inc., Valencia, CA, USA). DNA was analyzed by polymerase chain reaction (PCR) to detect PhHV-1 viral DNA utilizing primers amplifying a 450 bp region of the glycoprotein B (gB) (GenBank accession number U92270) gene sense: 5′-ACG TGA TGG AGC TCA TAC AGA AC-3′ and anti-sense: 5′-GCT AGC TCG TTG CTA ATC ATT GG-3′). Parallel reactions were run using primers (sense: 5′-GAA ACT GAT GAA GCT GAA CC-3′ and anti-sense: 5′-CTA AGA GGC AAA TGA CTT CCT CC-3′) amplifying a 544 bp fragment of the harbor seal ferritin gene (GenBank accession number AF246195) to control for the PCR amplificability of the DNA sample. Amplification conditions (MJ Research Thermocycler, MJ Research Inc., Waltham, MA, USA) used were 35 cycles with 40 s per step: denaturing at 94 °C, annealing at 60 °C and chain elongation at 72 °C. Amplified products were electrophoresed on a 1.5% agarose gel to visualize the results. Reaction sensitivity was determined by titration of a linearised plasmid clone containing the gB gene insert and by spiking mouse DNA with the plasmid clone, showing it was possible to detect a minimum of 166 copies of the viral DNA in samples. To confirm PCR specificity, selected positive samples (two mononuclear leukocyte, two oral, one nasal, one rectal, one ocular, one vaginal) from young and adult free-ranging and rehabilitating harbor seals were cloned and sequenced or direct sequenced by the chain termination method (Sanger et al., 1977).

Serum was harvested following centrifugation, frozen at −20 °C or lower, and analyzed using an ELISA for PhHV-1 specific IgG (King et al., 2001) in rehabilitating seals. All samples were tested in duplicate at a 1:100 dilution, and compared with a positive reference sample with a PhHV-1 specific antibody level designated at 100 units/ml. Results were reported as a percentage of this standard, and samples with antibody concentrations greater than 1 unit/ml were considered to be positive.

2.2. Temporal changes in PhHV-1 prevalence during rehabilitation (2000)

Thirty-five pups (30 pre-weaned, five recently weaned) were admitted for rehabilitation between 7 March 2000 and 29 September 2000. Seals were sampled every 2 weeks until the animals died or were released. Sequential mononuclear leukocyte, serum and swab (oral, nasal, rectal, ocular) samples were frozen for analysis until the end of the sampling season when samples were tested for evidence of PhHV-1 infection (viral DNA by PCR) and exposure (antibody by ELISA).

2.3. Monitored exposure under two different standard rehabilitation practices (2001)

Mononuclear leukocytes, serum and nasal swabs were collected from 10 pups (seven pre-weaned, three recently weaned) admitted between 1 March 2001 and 13 September 2001, and samples were analyzed weekly to determine when infected animals were present in the hospital. Three weaned seals (seals A–C) were admitted with detectable PhHV-1 DNA in the first week of June, and additional nasal swabs were collected from these individuals and placed into 1 ml complete Dulbecco's modified Eagles media (DMEM) (Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 50% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA,

Table 1
PCR (mononuclear leukocytes and nasal swabs combined) and ELISA detection of active PhHV-1 infections in three harbor seals (*P. vitulina*) (seals A–C) infected when admitted to The Marine Mammal Center, and the course of detectable viral DNA and antibody responses in seven harbor seals pups (seals D–J) believed to be naïve to herpesvirus exposure prior to contact with the infected seals (the shaded areas highlight the movement of infection through the hospital)

Days post-exposure	Separated pen				Direct contract pen						Adjacent pen 1				Adjacent pen 2					
	Infected A		Infected B		Infected C		Contact D		Contact E		Contact F		Contact G		Contact H		Contact I		Contact J	
	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
−10							−	−	−	−	−	−	−	−	−	−	−	−	−	−
−3	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
0	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
7	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
10	+	+	−	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
13	+	+	−	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
17	−	+	NS	+	−	+	Released		+	+	Released		−	−	−	−	−	−	−	−
20	−	+	NS	+	−	+			+	+			+	NS	Inc	−	−	−	−	−
23	NS	+	−	+	NS	+			+	+			+	+	+	−	−	−	Inc	−
30	−	+	−	+	−	+			−	+			+	+	+	+	+	−	+	−
37	−	+	−	+	−	+			Released				Released		NS	+	+	+	+	+
39	Released		Released		Released										−	+	NS	+	NS	+
42															Euthanized		NS	+	NS	+
44																		+	Released	
45																	Released			

Inc: inconclusive, no amplifiable DNA in sample; NS: no sample obtained.

USA) for virus isolation. Two of the infected animals (seals A and B) were housed in a pen separated from the others by a 0.9 m walkway. The third infected animal was clinically stable (seal C), and was placed in a pen in with two uninfected, clinically healthy pups being prepared for release (seals D and E) (Table 1), as commonly occurs under standard rehabilitation practices. Five other uninfected, clinically healthy pups were being housed in nearby pens, two (seals F and G) were housed in the pen adjacent to the pen containing seals C–E and three were in the next adjacent pen along the row (seals H–J) (Table 1). The pens were all separated by a 2 ft cement wall with chain link fence above, and each contained a pool that was maintained on a water filtration system that disinfects the outflow prior to recirculation. Animal husbandry (feeding, pen cleaning) procedures were performed by trained personnel that moved between pens while completing their daily routine. All seven uninfected animals (D–J) were approximately 1.5 months of age and believed to be naïve to previous PhHV-1 exposure, as all tested negative for PhHV-1 viral DNA by PCR and PhHV-1 specific antibodies by ELISA.

Blood and swab samples were collected every 3 or 4 days from all 10 animals and analyzed as described above to monitor for infection. The viral media from the nasal swabs was filter sterilized with 0.45 μm filters (Nalgene, Nalge Company, Rochester, NY, USA) and inoculated onto monolayers of Crandell Reese feline kidney cells (CrFK) that had been washed twice with complete DMEM. The flasks were incubated at 37 °C and rocked every 10 min for 1 h. Complete DMEM supplemented with 2% FCS was then added and the cultures were incubated and monitored daily for cytopathic effect (CPE). Cells derived from cultures showing cytopathic effect were submitted for electron microscopy as well as for subsequent PhHV-1 PCR and sequencing.

2.4. Comparison of PhHV-1 prevalence among free-ranging and rehabilitating harbor seals

Free-ranging seals [$n = 270$; 19 pre-weaned pups, 102 post-weaned pups (2–11 months), 149 seals older than 1 year (yearlings, subadults, adults)] were captured from March 1999 to March 2002 in South Humboldt Bay ($n = 17$), Point Reyes National Seashores (Double Point and Point Reyes Headlands; $n = 55$), San Francisco Bay ($n = 26$) and Monterey Bay (Elkhorn Slough, San Lorenzo River and Pebble Beach; $n = 172$) using tangle nets, a purse seine net or salmon nets (Jeffries et al., 1993). Upon capture measurements, examinations and sample collection were performed as described for rehabilitating seals above. The presence of detectable PhHV-1 viral DNA in clinical samples obtained from free-ranging harbor seals was analyzed with respect to age, sex and location. Comparisons were also made between the timing of detecting PhHV-1 DNA in samples collected from free-ranging seals and the time point at which rehabilitating harbor seals first tested PCR positive.

2.5. Analysis

The associations between detecting viral infections and age, sex and season of infection of free-ranging seals were evaluated using either a χ^2-test for association (Fleiss, 1981) or Fisher's Exact test (Fisher, 1935), and odds ratios (OR) with 95% confidence intervals (CI) were calculated to estimate the strength of these associations. The association between the clinical sample type and the likelihood of obtaining DNA from that sample type or of that sample type testing positive was also evaluated using a χ^2-test for association or Fisher's Exact test (Epi Info® 2000 software, Version 1.1.2, June 2000, Centers for Disease Control and Prevention, Atlanta, GA, USA and Medcalc® Statistical software, Version 6.0, 1993, Mariakerke, Belgium).

3. Results

3.1. Temporal changes in PhHV-1 prevalence during rehabilitation (2000)

The majority of pups admitted early in 2000 (March–early May) were PhHV-1 seronegative (22/27) (Fig. 1). At admission, five of the 27 animals had low levels of antibody that declined to very low levels within 12 days. All but two pups (25/27) had no detectable viral DNA by PCR in clinical samples until the middle of May. These two seals were premature and tested positive by PCR at a single sampling point early during their rehabilitation (early March and early

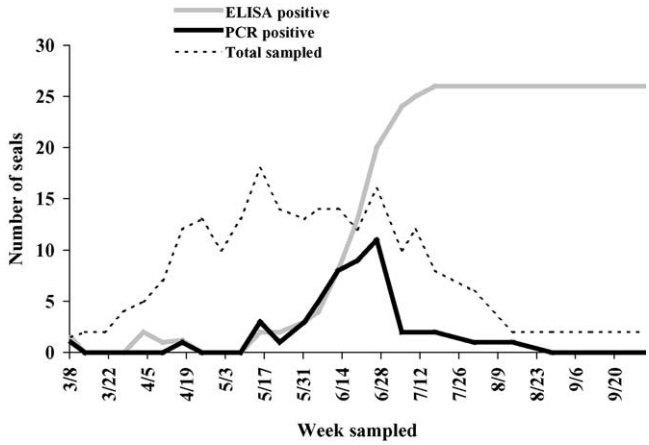

Fig. 1. Evidence of exposure to PhHV-1 followed by seroconversion in harbor seals pups at The Marine Mammal Center in 2000 ($n = 35$). The curves represent the total number of animals sampled biweekly, the number with detectable viral DNA by polymerase chain reaction (PCR) and the number that tested antibody positive prior to the beginning of May and the cumulative number that had seroconverted and remained antibody positive until released as measured by enzyme linked immunosorbent assay (ELISA) at each sampling date.

April); however, neither became clinically ill nor seroconverted at this time. In the middle of May, three animals were admitted that had PCR detectable PhHV-1 DNA in mononuclear leukocytes and/or in swab samples; two of which were seropositive. Within the next 10 days, the first of the harbor seals housed near these infected animals tested virus positive by PCR and seroconverted by the next sampling date (within 14 days). Within 1 month 78% (14/18) of the previously viral negative animals had detectable viral DNA in clinical samples and seroconverted over the next 14 days. The number of pups that were viral positive by PCR was declining by the end of July, and all animals had seroconverted (26/26) and remained seropositive until they were released (final release at the end of September). These animals remained clinically healthy during this period of exposure to PhHV-1, and 83% (29/35) of admitted pups were released back into the wild. Animals that were released ($n = 3$) or died ($n = 6$) early in the season and did not have contact with seropositive infected animals, did not seroconvert, nor did they have any detectable viral DNA present in clinical samples. The two premature seals that tested PCR positive at a single sampling point 10 and 4 weeks prior to admittance of the three viral positive individuals subsequently exhibited the same timing and pattern of testing virus positive and then seroconverting as their naïve conspecifics.

3.2. Monitored exposure under two different standard rehabilitation practices (2001)

During 2001, all admitted animals tested negative for both PhHV-1 viral DNA and antibodies from March through the end of May. Active PhHV-1 infections detected by PCR in the three seals (pups A–C) admitted in early June were confirmed by virus isolation. The characteristic cytopathic effect (Gulland et al., 1997) was seen within 3 days of incubation of media from nasal swab samples. Electron microscopy of infected cell cultures also revealed enveloped (110–111 nm) and non-enveloped (108–109 nm) herpes-like viral particles, as previously described (Gulland et al., 1997). Additionally, PCR and sequencing of the gB fragment from DNA extracted from these cell cultures yielded identical sequences to those reported previously by King et al. (1998), again confirming the presence of infection with PhHV-1. Following exposure to PhHV-1 by direct contact with infected pup C (Table 1), contact animal E tested positive for viral DNA in mononuclear leukocytes within 4 days, in nasal secretions within 7 days and had detectable antibodies at 9 days following the first PCR positive test. Viral infection remained detectable by PCR for 19 days in this animal. Contact seal D tested PCR positive in mononuclear leukocytes within 7 days and in nasal secretions within 10 days post-exposure, and was released to the wild 8 days later due

to the onset of stress behavior incompatible with captivity. Seroconversion could not be documented in this seal prior to release, but it remained PCR positive for viral DNA until released. Seal G, in adjacent pen 1, tested positive for PhHV-1 DNA in both mononuclear leukocytes and nasal secretions within 20 days following the direct exposure of seals D and E; animals in adjacent pen 2 became PCR positive in both mononuclear leukocytes and nasal secretions within 23 and 30 days (seals H–J; Table 1). Seroconversion occurred on average approximately 7 days following detection of viral DNA in these indirectly exposed seals. All animals were clinically healthy at the time of exposure, and no clinical signs of illness were observed during the study. All animals were released, with the exception of one seal (seal H) that was euthanized for an unrelated problem.

3.3. Comparison of PhHV-1 prevalence among free-ranging and rehabilitating harbor seals

Of the 121 free-ranging pre- and post-weaned pups that were tested, 49 (40%) were PCR positive for PhHV-1 DNA, whereas only four of the 149 (3%) animals greater than 1 year old were PCR positive ($P < 0.001$) (Fig. 2). Young harbor seals were 23 times more likely (OR = 23.02, 95% CI = 7.99–66.36) to have detectable PhHV-1 viral DNA in clinical samples than adults, and the majority tested positive during the spring and early summer in all 3 years ($P < 0.001$). This seasonal trend of viral infection in the young harbor seals was observed at all capture locations. The PCR positive older animals were two adult females and two yearling females that tested positive in late summer, fall or early winter. No significant association was found between sex and a detectable viral infection in the young animals ($P = 0.49$) or in those older than 1 year ($P = 0.12$). However, the trend toward older females rather than males being more likely to be PCR positive is noteworthy, since very few adults tested positive. Similar to the free-ranging seals, 54% (46/85) of rehabilitating seals sampled from 1999 to 2001 tested positive for PhHV-1 viral DNA. As with the free-ranging seals, most PCR positive individuals were pre- or post-weaned pups with the exception of two adult females and one subadult male, all three of which were ill or injured. The timing of infection seen in the rehabilitating seals also corresponded well with that observed in the free-ranging harbor seals, occurring mostly from May through July. The majority of the 270 free-ranging harbor seals that were captured and examined appeared to be clinically healthy, as wounds or evidence of illness were only noted for 33 animals.

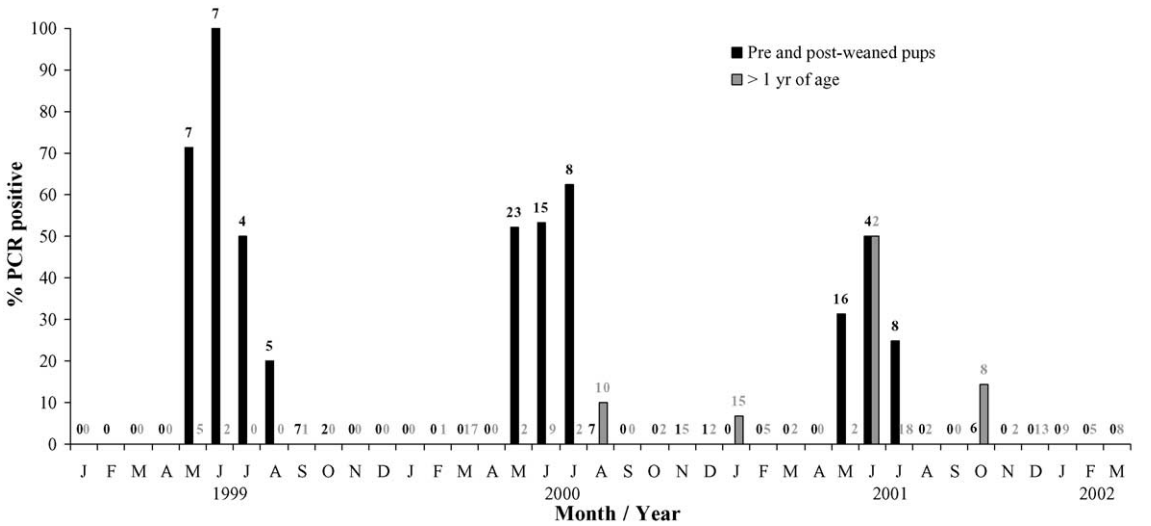

Fig. 2. Prevalence of PhHV-1 viral DNA PCR in clinical samples from pre- and post-weaned free-ranging harbor seal (*P. vitulina*) pups ($n = 121$) and harbor seals older than 1 year ($n = 149$) along the central California coast March 1999 to March 2002. The numbers on the bars represent the total number of animals sampled each month for both age groups.

We further evaluated these 33 animals in order to determine whether free-ranging harbor seals that were hurt or ill were more likely to test positive for PhHV-1 DNA. Overall, no significant association was found between disease state and viral DNA as only 18% (6/33) of the injured or ill animals had detectable PhHV-1 viral DNA in clinical samples ($P = 0.99$). However, when adjusting for age a significant association was found in seals older than 1 year of age, as animals in this group that were ill or inured were more likely to test PCR positive ($P < 0.001$). One of the two adult females and both yearling females that tested positive fell into this group.

A total of 2052 samples were collected and analyzed for viral DNA from rehabilitating and free-ranging harbors seals combined (Fig. 3). DNA of quality compatible with PCR analysis (as shown by successful amplification of the ferritin control PCR) was obtained from 99% (741/749) of the mononuclear leukocyte samples, and from 65% of the nasal (421/652), 72% of the oral (265/370), 65% of the rectal (153/237), 51% of the vaginal (20/39) and 60% of the ocular (3/5) swabs. No difference was found among sample types for the likelihood of extracting DNA ($P = 0.73$). Overall, nasal swabs most commonly tested positive for PhHV-1 DNA (116/408), followed by oral swabs (39/265), mononuclear leukocytes (93/741), rectal swabs (17/153), ocular swabs (1/3) and vaginal swabs (1/20). Accordingly, nasal swabs were the best clinical sample type for identifying infected pre- and post-weaned seals as they most commonly tested positive for PhHV-1 DNA ($P < 0.001$; Fig. 3). Phocine herpesviral DNA from animals older than 1 year was less likely to be detected in mononuclear leukocytes compared to swab samples ($P = 0.014$), with nasal swabs again being the most common sample type testing positive. PCR positive samples representing all animals and collection sites were identical (8/8) in gB nucleotide sequence, as well as to the previously published gB fragment (King et al., 1998), confirming that the PCR assay was obtaining the correct target amplicons.

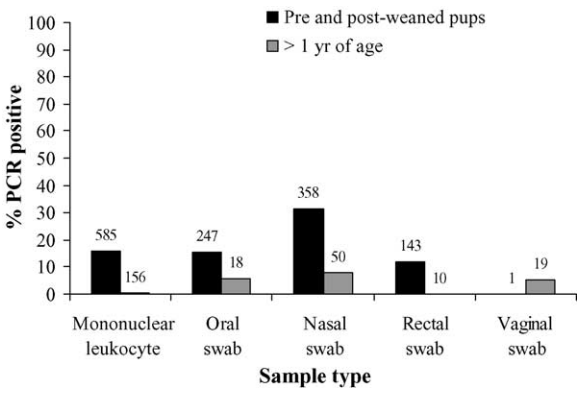

Fig. 3. Prevalence of PhHV-1 DNA detected by PCR in different sample types (mononuclear leukocytes; oral, nasal, rectal and vaginal swabs) in harbor seals (*P. vitulina*) (rehabilitating and free-ranging combined) less than and older than 1 year of age collected from March 1999 to March 2002. The numbers on the tops of bars are the number of samples that were tested in each group.

4. Discussion

Active PhHV-1 infections appear to be common in young harbor seals in California, and seals can successfully mount an immune response and survive PhHV-1 infection. The timing of infection appears to be seasonal, occurring in spring and early summer, which also correlates with the timing of pupping and weaning of Pacific harbor seals in this area. Mating follows pupping, and in California, pups are typically born between March and late May (Bigg, 1969a, 1969b), although premature pups can be born as early as February (King, 1983; Tempte et al., 1991), coinciding with the beginning of the season in which pups are stranded and admitted for rehabilitation. Weaning takes place over approximately a 1 week period and begins, on average, when the pups are 30 days of age. Thus, by the end of June the majority of pups born in California are weaned (Bigg, 1969b; Boulva and McLaren, 1979), and by July few pup strandings are observed. Since the largest number of young, infected pups both rehabilitating and free-ranging were detected annually from May to July, this seasonality is likely due to a combination of factors. This time period corresponds to the season when there is the largest number of susceptible naïve animals, i.e. newborn pups, on the rookeries that can be exposed to other seals shedding the virus. This timing also coincides with the breeding and mating season, which can be stressful for adults, therefore leading to a source of virus due to recrudescent infections.

Seroconversion was observed in rehabilitating seals following the detection of PhHV-1 DNA by PCR in mononuclear leukocyte and swab samples. This suggests that the animals had active infections and were viremic and/or shedding the virus during these periods. The monitored exposure study (2001) showed viral shedding occurred 4–7 days post direct contact exposure, and between 20 and 30 days following the initial opportunity for indirect contact and exposure. These animals had detectable PhHV-1 DNA in clinical samples for between 7 and 19 days, and seroconversion followed evidence of infection within 7–9 days.

Two interesting patterns were noted early in the lives of the rehabilitating seals during 2000. First, five pups between approximately 1 and 3 weeks of age had low levels of rapidly declining PhHV-1 specific antibodies. Previously documented by King et al. (2001), this finding is consistent with the presence and metabolism of passively transferred maternal antibodies commonly observed in other neonates (Tizard, 2000). Second, two premature seals tested positive for viral DNA at a single sampling point at a time (early March and early April) when all the other rehabilitating seals appeared to be naïve. The two did not permanently seroconvert in the following days as would have been expected. Interestingly, both of these animals had had previously measurable (presumably maternal) antibodies by ELISA that waned prior to or during the period in which they tested PCR positive. It is probable that these animals became infected during gestation or parturition, as can occur in other species (Roizman et al., 1981) and may have been experiencing a reactivated infection. It is notable that these animals did not mount a detectable antibody response following this evidence of infection as harbor seals are thought to be immunocompetent at this age (Ross et al., 1994). A possible explanation for this lack of immunologic response is that the presence of maternal antibody prevented production of antibody by the pups. Alternatively, immunotolerance may have occurred as a result of fetal infection, but since seroconversion occurred later during rehabilitation the former explanation is more likely.

The monitored exposure study (2001) provided evidence that PhHV-1 can be rapidly transmitted horizontally between harbor seal pups that are in direct contact probably via nasal and oral secretions. This finding is supported by nasal swabs being the most common sample to test PCR positive for viral DNA, followed by oral swabs and then mononuclear leukocyte samples. This route of transmission is thought to be common for other alpha herpesviruses, such as canine herpes and herpes simplex (Roizman et al., 1981; Carmichael and Greene, 1990). The animals in this study (2001) that acquired the infection indirectly likely did so through contact with contaminated equipment or personnel, or possibly via respiratory droplets. There was some variability among animals from the time of first exposure or exposure opportunity to infection, as well as for the length of time that they remained viremic and/or shed the virus. This variability may be due to the viral load to which the pup was exposed, to route of exposure, and/or individual differences in immune function.

In addition to the two premature newborns with detectable early infections, our studies provided additional evidence for the potential for vertical transmission between mother and newborn pup, as four adult female harbor seals (two undergoing rehabilitation and two free-ranging) were found to be shedding the virus in nasal secretions and in vaginal secretions. Although these females could have been recently infected, it is also possible that latent infections occur with recrudescence during periods of physiologic stress, as is common in other herpesviral infections (Roizman, 1982). Both adult females undergoing rehabilitation that tested positive were ill or injured as well as peri-parturient, and the two free-ranging adult females were most likely pregnant, as they were tested after the mating season in August and October and pregnancy rates for seals are high in this area (Greig, 2002). Studies in humans have shown that nearly 1% of pregnant women may have recurrent herpesvirus infections in the absence of any clinical signs, and provide a source of virus for newborns (Hatherley et al., 1980). Since apparent pregnancy associated immune suppression has been previously demonstrated in phocids (King et al., 1994), viral reactivation could occur in pregnant harbor seals.

Significantly more free-ranging harbor seal pups tested PCR positive for PhHV-1 than older animals. This large number of infected pups around the time of weaning correlates with a previous study that showed that the prevalence of PhHV-1 specific antibodies was significantly higher in post-weaned pups compared to

pre-weaned pups, suggesting that the majority of pups were exposed to the virus during weaning (Goldstein et al., 2003). These data parallel results from humans infected with Herpes Simplex-1, in which viral shedding occurred in 20% of children 7 months to 2 years old, and only in 2.7% of individuals over 15 years of age (Buddingh et al., 1953; Douglas and Couch, 1970).

Previous studies (Osterhaus et al., 1985; Gulland et al., 1997; King et al., 2001) have shown high morbidity and mortality due to PhHV-1 infections in seals during rehabilitation, but such outbreaks did not occur at The Marine Mammal Center during this study. A new hospital for the care of harbor seal pups was completed at the end of 1998. The new facility was designed to decrease the necessity for handling of the animals in order to reduce stress. Additionally, animal husbandry changes were made, such as an improved diet and increased daily caloric intake. A decrease in PhHV-1 related deaths was also noted at a rehabilitation center in The Netherlands following isolation of the virus and similar revision of husbandry protocols (Harder et al., 1997). In both cases this decrease in mortality may be due in part to better veterinary care and quarantine measures.

5. Conclusions

These data show that PhHV-1 infections occur commonly in young harbor seals in California, USA, during the spring breeding and pupping season. Although the disease may be fatal in animals that are sick or under stress, healthy seals can be infected without showing clinical signs. Transmission can occur by direct and indirect contact, and can result in seroconversion without clinical disease. The role of stress in increasing the severity of PhHV-1 associated morbidity and mortality in free-ranging seals is unknown. Therefore, it may be beneficial for animals in rehabilitation to undergo natural exposure to infected seals under seasonally appropriate, controlled conditions when individuals are clinically healthy. This controlled exposure may result in immunity prior to potentially dangerous exposure at more stressful times following release. The cycle of PhHV-1 infections in harbor seal populations is most likely maintained via vertical transmission from mother to newborn in a small proportion of animals and horizontally through direct contact via oro-nasal secretions in the majority of pups around weaning. Maintenance of population prevalence is probably due to reactivated infections annually in adult and subadult harbor seals.

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