Widespread neonatal infection with Phocid Herpesvirus 1 in free-ranging and stranded grey seals (*Halichoerus grypus*)

Running page head: PhHV1 in grey seals

Author list:

Johanna L. Baily[a,b,c*], Kim Willoughby[a], Madeleine Maley[a], Jacob Chapman[a], Romain Pizzi[d,e], Ailsa J. Hall[b] and Mark P. Dagleish[a]

[a] Moredun Research Institute, Edinburgh, Scotland, EH26 0PZ, UK

[b] Sea Mammal Research Unit, Gatty Marine Laboratory, University of St. Andrews, St. Andrews, Fife, KY16 8LB, UK

[c] Present address: Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[d] Royal Zoological Society of Scotland, 134 Corstorphine Road, Edinburgh, EH12 6TS, UK

[e] Scottish SPCA National Wildlife Rescue Centre, Fishcross, Alloa, Clackmannanshire, FK10 3AN, UK

* Corresponding author:

Johanna L. Baily

Email: j.l.baily@stir.ac.uk

Accepted for publication in *Diseases of Aquatic Organisms* published by Inter Research. The final published version is available at: https://doi.org/10.3354/dao03345

Abstract

Phocid herpesvirus 1 (PhHV-1) is known to infect grey seals (*Halichoerus grypus*) but little is known about its pathogenicity or true prevalence in this species. To investigate the prevalence of and risk factors associated with PHV-1 infection, nasal swabs were collected from grey seal pups and yearlings on the Isle of May, a well-studied grey seal breeding colony, and from stranded grey seal pups submitted to a rehabilitation centre. Phocid herpes virus 1 nucleic acids were detected in nasal swabs from 58% (52/90) of live, free-ranging grey seal pups, 62% (18/29) of live, stranded grey seal pups and 28% (5/18) live free-ranging yearlings suggesting recrudescence in the latter. Location within the colony, pup body mass and stranding were determined to be risk factors for shedding PhHV-1 in live seal pups with a significantly higher prevalence of PhHV-1 in pups born on the tidal boulder beach when compared to other sites; a significantly positive correlation of PhHV-1 shedding and pup body mass and a higher prevalence in stranded grey seal pups compared to their free-ranging conspecifics. The prevalence of PhHV1 in dead pups on the Isle of May was 56% (27/48) with a positive PhHV-1 PCR status significantly associated with hepatic necrosis ($p=0.01$), thymic atrophy ($p<0.001$) and buccal ulceration ($p=0.027$). Results indicate that PhHV-1 was widespread in the pups in the Isle of May grey seal breeding colony.

Keywords: Grey seals; *Halichoerus grypus*; Herpesviridae; free-ranging; rehabilitation

Introduction

Phocid herpesvirus-1 (PhHV-1) is an alphaherpesvirus which infects both grey (*Halichoerus grypus*) and harbour (*Phoca vitulina*) seals in the Eastern Atlantic and Pacific Oceans (Borst et al. 1986, Harder et al. 1996, Gulland et al. 1997, Martina et al. 2002, Himworth et al. 2010). The alphaherpesvirus subfamily consists of large double-stranded DNA viruses of variably narrow to broad host range, typically characterised by rapid replication in cell culture, lytic infection and the ability to develop latent infections in sites such as the trigeminal ganglia (Caswell & Williams 2007). Alphaherpesviruses are responsible for a number of economically important diseases in domestic species including Bovine herpesvirus 1 (BoHV-1) which causes infectious bovine rhinotracheitis in calves and abortion in cows, Suid herpesvirus which causes Aujeszky's disease and abortion in pigs and Equid herpesviruses 1 and 4 which result in respiratory and neurological diseases in horses. Another feature of many alphaherpesviruses is the ability to induce immunosuppression (Winkler et al. 1999, Brukman & Enquist 2006, Van de Walle et al. 2008).

Infection of harbour seals by PhHV-1 and the resultant disease has been well documented as the pathology and pathogenesis of this virus differs between geographically distinct populations. Eastern Atlantic harbour seals develop hepatic necrosis, interstitial pneumonia, renal tubular epithelial degeneration, oral mucosal ulceration and lymphodepletion (Borst et al. 1986), whereas Pacific harbour seals develop adrenocortical necrosis and multifocal hepatic necrosis with intranuclear viral inclusion bodies (Gulland et al. 1997). Morbidity and mortality due to PhHV-1 vary from high (Osterhaus et al. 1985, Gulland et al. 1997) to low (Goldstein et al. 2004) suggesting that extrinsic factors, such as stress or concurrent disease, may affect the severity of the clinical manifestation of the disease in harbour seals (Goldstein et al. 2004). However, very little is known about the pathogenicity and epidemiology of

PhHV-1 infection in grey seals. Grey seals develop less severe clinical signs upon PhHV-1 infection than harbour seal pups when in rehabilitation centres (Martina et al. 2002). In Harbour seals, the virus causes moderate to severe clinical respiratory disease in younger pups, with more mild disease and correspondingly less severe clinical signs to sub-clinical infections in older animals (Martina et al. 2002).

"Stranded" seal pups are assumed to be stressed and are frequently malnourished, both factors known to impair immune function (Snyder 2007) and this would result in pups being more susceptible to infection and prolong shedding. Consequently, we hypothesise that the prevalence of known enzootic grey seal pathogens, such as PhHV-1, is likely to be higher in stranded seal pups than in seal pups from their natal colonies and higher in dead seal pups compared to live pups.

The aims of this study were to assess the prevalence and risk factors for PhHV-1 infection in live free-ranging and stranded grey seal pups and in yearlings. Within a breeding colony, the effect of location within the colony (ground substrate and animal movements), time of birth during the pupping season and host factors such as body mass and age were investigated as risk factors for the presence of PhHV-1. In addition, the prevalence and pathology associated with PhHV-1 infection were investigated in dead grey seal pups both on their natal colony and in a rehabilitation centre.

Materials and methods

Animals and sampling

Grey seal pups and yearlings were sampled during the pupping season of autumn 2011. Free-ranging, live (n=90) and dead (n=50) grey seal pups and live yearlings (n=19) were sampled on the Isle of May (IOM), Firth of Forth, UK. Live grey seal pups were sampled from three distinct sites on the Isle of May which had different substrate and animal movement characteristics (tidal boulder beach with twice daily congregations of pups due to displacement by the tide (n=30); muddy/grassy slope with sedentary pups (n=30) and stagnant rocky pools with sedentary pups (n=30)) and at three different time points (early, mid and late pupping season). Dead grey seal pups were collected opportunistically throughout the season from varied sites on the colony and within 48h of death. Additionally, live-stranded grey seal pups (n=31) were sampled within 24h of arrival at the Scottish Society for the Prevention of Cruelty to Animals National Wildlife Centre (SSPCA), Fife as part of the routine health assessment procedure to determine suitability for care and rehabilitation prior to release back into the wild. All sampling of live animals on the Isle of May was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures) Act, 1986. All sampling of live animals submitted to the rehabilitation centre was for diagnostic purposes to determine the cause of stranding and future treatment regime.

In addition, stranded pups at the SSPCA rehabilitation centre that subsequently died or were euthanised on humane grounds (n=9) were sampled also as part of this study.

The following data were recorded: sex, pup developmental stage (stage I to stage V) as described by Kovacs and Lavigne (1986), body mass (to the nearest 100g) using spring balanced scales (Salter Industrial Measurements Ltd., West Bromwich, UK) in a pup-bag; length (nose to tail, to the nearest 5mm), girth immediately posterior to the axilla (to the

nearest 5mm) and the presence of any external anomalies (bites, areas of alopecia, nasal discharge, ocular discharge, presence/absence of umbilical cord).

A nasal swab was obtained from all live and dead animals examined, using a nylon flocked swab and placed into universal transport medium (UTM) (swab and UTM: Catalogue no. 346C, Sterilin, Newport, UK). Swabs were initially stored at 4°C and subsequently frozen at -80°C within 12 hours of collection until analysis. A full post-mortem examination was performed on all dead pups and samples collected for histopathology. Pooled representative samples of 10 organs (liver, spleen, mesenteric lymph node, ileo-caeco-colic junction, kidney, left cranial lung lobe, bronchial lymph node, right ventricle, tonsil and brain) were collected aseptically, placed in a sterile gentleMACS™ M tube (Miltenyi Biotec, Bisley, UK) and frozen at -80°C until analysis.

Sample processing and analysis

Nasal swabs in universal transport medium were placed into a sonicator bath for 30s and then centrifuged at 2000g for 10min at 4°C. The resultant supernatant was stored at -80°C until analysis. Pooled tissue samples were homogenised with 5ml viral transport medium using a Dispomix homogeniser (Miltenyi Biotec Ltd., Bisley, UK), centrifuged at 2000g for 10min at 4°C and the resultant supernatant stored at -80°C until analysis. Nucleic acids were extracted from supernatants using the NorDiagViral NA Arrow automated extraction robot (Alere, Stockport, UK) as per manufacturer's instructions and stored at -20°C. Extraction controls (water or PBS) were processed with every batch of tissue and swab samples.

Detection of PhHV-1 specific nucleic acids

Nucleic acids extracted from the nasal swabs and pooled tissue samples were assessed for the presence of PhHV-1 nucleic acids using an end-point PCR assay amplifying a 450bp fragment of the PhHV-1 specific glycoprotein B (gB) gene as described previously (Goldstein et al. 2004). DNA extraction controls, positive and negative (no template DNA) controls were included for all reactions. Positive control material consisted of grey seal liver, previously found positive for PhHV-1 following pan-Herpesvirus nested degenerate PCR (Ehlers et al. 1999) and subsequent direct sequencing of the product (Eurofins MWG, Ebersberg, Germany). Reactions were performed in a total volume of 50 µl, using Platinum Taq (Invitrogen, Paisley, UK) and containing a final concentration of 200 µM of each primer and 2 µl of template DNA. Reactions were performed in a Techne Workbench thermal cycler (Techne, Stone, UK) with the following cycling parameters: 95°C for 3 min (denaturation/Taq activation) followed by 35 cycles of denaturation for one minute at 94°C, annealing for one minute at 60°C and extension for one minute at 72°C with a final extension at 72°C for 10 min. Reaction products were electrophoresed on a 1.5% agarose gel, stained with Sybrsafe (Invitrogen) and visualised by UV transillumination using a gel doc system (Alpha Imager, San Leandro, CA). A reverse transcriptase real-time PCR (RT-qPCR) assay amplifying the stable housekeeping gene beta-actin was used in a separate assay to assess sample integrity as described by Thonur et al. (2012).

Histopathology and immunohistochemistry

Samples from 59 pups (50 from IOM and 9 from SSPCA) were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax for histopathological examination by a boarded veterinary pathologist. After review of histopathological findings, the degree of thymic atrophy (as a proxy for immunosuppression) was assessed using a

subjective scoring system ranging from 0 to 3 (none, mild, moderate and severe respectively). The adrenal cortico-medullary ratio was measured (AnalySIS Five software, Soft Imaging System GmbH, Münster, Germany). A cause of death was assigned to each animal by a boarded veterinary pathologist following gross post mortem, detailed histopathological examination and routine bacteriology examination.

An attempt at immunohistochemical localisation of PhHV-1 within microscopical lesions was made in the present study using antibodies directed against both BoHV-1 and FeHV-1 in separate assays (Baily 2014), and no cross-reactivity of PhHV-1 was detected with these antibodies on control tissue slides (harbour seal infected with PhHV1). Briefly, endogenous peroxidase activity was blocked by immersion in 3% H2O2 in methanol (vol/vol) for 20 min. Tissues were incubated with 150 µl of 25% normal goat serum (NGS; Vector) in PBS-0.05% Tween 20 (PBST) for an hour at room temperature to block non-specific antibody binding. Primary antibodies were Bovine herpesvirus 1 (clone F2; mouse monoclonal IgG2b (Veterinary Medical Research and Development, Pullman, WA, USA), dilution 1:5000; incubated overnight at 4°C), and feline herpesvirus 1 (Clone FHV5; mouse monoclonal IgG2b (Acris antibodies, Herford, Germany), dilution 1:100, incubated 30 minutes at room temperature). Visualisation of the primary antibody was by Envison™ anti-mouse HRP polymer (Vector Laboratories etc.) as per manufacturer's protocols. Positive control material consisted of bovine liver infected with BoHV1 and feline lung infected with FHV1 respectively and showed immunolabelling within lesions.

Statistical analyses

Prevalence data were analysed using the R statistical software package (R Core Team 2013). To investigate significant differences in prevalence of PhHV-1 in nasal swabs between

groups Fisher's exact tests were performed. For univariate analysis generalized linear models (GLMs) with a binomial distribution family and a logit link function was used to evaluate the effects of different morphologic measurements (standard length, body mass, axillary girth), age (stage of pup development as a proxy), sex, sampling time, colony (stranded versus free-ranging animals) and ground substrate on the presence or absence of PhHV-1. Multivariate logistic regression was performed using R with a forward stepping algorithm and a p value of ≤ 0.05 for inclusion in the model based on the likelihood ratio test. Any stranded grey seal pups which died at the rehabilitation centre were excluded from the multivariate analysis of PhHV-1 nasal swab status to avoid repeat sampling of the individual animals swabbed on entry to the rehabilitation centre. As length, body mass, girth and pup stage were highly correlated with each other, and so as not to violate the assumption in generalised linear models concerning the independence of independent variables, body mass was chosen as the most reliable, independently verified and reproducible of the morphometric measurements in the present study and retained for further analyses. Akaike's Information Criterion (AIC) was used to compare models and choose the most parsimonious for each variable. Residual analysis was used to assess goodness of fit.

Results

Prevalence of PhHV-1 in grey seal pups and yearlings

The prevalence of PhHV-1 in nasal swabs of all pups was 59.6% (105/176) with a prevalence of 62% (18/29) in stranded, live pups presented for rehabilitation, 57.8% (52/90) in free-ranging live pups on the IOM colony and 56.3% (27/48) dead pups on the IOM colony. The group with the highest prevalence was the stranded dead pup group with a prevalence of 89% (8/9, Table 1). Yearlings had a significantly lower prevalence of PhHV-1 (28%) than pups (60%) ($p=0.008$; Fisher's exact test).

Odds ratios for categorical risk factors were determined for PhHV-1 (Table S 1). Live and dead free-ranging pups sampled in the late pupping season had significantly higher odds of being PhHV-1 positive in nasal swabs, as determined by presence of PhHV-1 nucleic acids, than pups sampled in the early pupping season ($p<0.001$, GLM). Also, free-ranging pups sampled at stage I (youngest age sampled) were significantly less likely to be shedding PhHV-1 in nasal secretions compared to pups in any of stages III, IV and V ($p=0.004$; $p=0.002$; $p=0.024$, respectively).

For live, free-ranging pups, the final logistic regression model demonstrated that an increased risk for PhHV-1 nasal shedding was associated with the location on the colony, as well as with the body mass of the pup at the time of sampling (Table S 2). Seals born around the rocky pools (OR=0.55, 95% CI 0.18, 1.62, $p=0.015$) and on the grassy, muddy slope (OR=0.24, 95% CI 0.08, 0.72, $p=0.002$) were significantly less likely to be positive for PhHV-1 in nasal swabs than pups born on the tidal boulder beach. The odds of shedding PhHV-1 were increased by a factor 1.15 for every extra kg of pup body mass and an increase in pup body mass from 10kg to 20kg results in an increase in odds of 3.91of having PhHV-1 specific DNA on the nasal mucosal membrane.

When considering all live pups (stranded and free-ranging), stepwise logistic regression analysis indicated that, among the variables examined, body mass and colony (stranded versus free-ranging animals) were statistically significant multivariate predictors of positive PhHV-1 PCR result from nasal swabs. The final logistic regression for PhHV-1 nasal swab PCR status is presented in Table S 3. No correlation of PhHV-1 with body mass, length or girth was found in the samples from yearlings.

Tissue samples

The presence of PhHV-1 DNA in nasal swabs correlated significantly with the presence of PhHV-1 DNA in pooled tissue samples ($p=0.005$). No significant difference was noted between the prevalence of PhHV-1 in tissues of dead free-ranging pups on the IOM when compared to dead stranded pups at the SSPCA.

Pathology

To investigate the relationship between PhHV-1 and the presence of specific lesions in dead pups, those with a positive result in either a nasal swab or pooled tissue sample were considered positive for PhHV-1. A positive PhHV-1 PCR result was significantly associated with the presence of hepatic necrosis (FET, $p=0.01$), thymic atrophy (FET, $p<0.001$) and buccal ulceration (FET, $p=0.027$) (Table S 4). The degree of thymic atrophy was significantly associated with the PhHV-1 status of the pups examined with a higher prevalence of PhHV-1 in pups with moderate to severe thymic atrophy (Figure 1). In addition, the presence of thymic atrophy was positively correlated with adrenal cortico-medullary ratio ($p=0.006$). A single pup presented intranuclear inclusion bodies within hepatocytes in proximity to foci of necrosis. No inclusion bodies were noted in the adrenal glands of any pups examined. There was no relationship between PhHV-1 infection and interstitial pneumonia or encephalitis.

Discussion

This is the first study of the prevalence of PhHV-1 in live and dead grey seal pups on a specific breeding colony. The widespread presence of PhHV-1 in live and dead grey seal pups (56.3% and 57.8% in dead and live free-ranging grey seal pups, respectively) shows that this virus is widespread on the Isle of May breeding colony and suggests that most pups will have been exposed to this virus by the time they leave the island (at approximately 6 weeks of age). This finding is typical of host-adapted alpha herpesviruses in densely populated breeding colonies, such as is seen in breeding catteries with felid herpesvirus 1 (FeHV-1) and breeding kennels with canid herpesvirus 1 (CaHV-1) (Gaskell & Willoughby 1999, Gaskell et al. 2007) and is comparable to the high prevalence of PhHV-1 (40%) in nasal swabs recorded in live, free-ranging pre- and post-weaned harbour seal pups in California (Goldstein et al. 2004) and 91 to 93% seroprevalence of PhHV-1 in adult grey seals (Roth et al. 2013).

The strong correlation between PhHV-1 DNA in nasal swabs and increased pup body mass, itself confounded with age, suggests horizontal transmission within the colony. None of the stillborn pups (n=5) were positive for PhHV-1, but the small sample size prohibits further interpretation regarding whether vertical transmission occurs. In other animal species infected with alphaherpesviruses, such as CaHV-1and FeHV-1, transmission often occurs at or around parturition, with recrudescence of the virus in maternal tissues and transmission to the neonate via vaginal secretions or nasal shedding (Gaskell & Willoughby 1999, Schlafer & Miller 2007). Goldstein et al. (2004) showed that viral shedding in nasal secretions of stranded harbour seal pups occurred 4–7 days post direct contact exposure. These findings could explain the low prevalence of nasal shedding in early stage pups in the present study.

Indeed, a negative PCR result in nasal swabs in early stage pups would not rule out the possibility of peri-natal transmission within mother-pup pairs.

Given the non-invasive nature of the study, it was not possible to assess tissue presence of PhHV-1 in live pups. However, the number of pups infected with PhHV-1 is likely to be substantially higher than the number of pups shedding the virus in nasal secretions. As a crude measure, if one assumes that stage II and stage V pups are of sufficiently different ages (average 4 days vs 18 days respectively) and that the duration of PhHV-1 nasal shedding does not exceed 14 days (7-19 days according to Goldstein et al. (2004)), shedding in stage V pups is likely to represent newly infected pups. The combined prevalence of PhHV-1 in stage II (39%) and stage V pups (66.7%) found in this study would support a very high risk of exposure of pups to the virus.

The significantly higher prevalence of PhHV-1 among live, free-ranging pups on the tidal boulder beach site compared to the other two sites may be a consequence of regular displacement and subsequent crowding of these pups due to the twice daily high tides. This may lead to higher stress levels and also increased contact between pups and other adult seals. The very high prevalence of PhHV-1 found in pups dying at the rehabilitation centre (88.9%) may result from stress-induced viral replication and/or horizontal transmission of virus within individuals at the rehabilitation centre, such as that described previously in harbour seals (Goldstein et al. 2004, Himworth et al. 2010). The role of stress in the spread and pathogenesis of PhHV-1 warrants further investigation as the comparable prevalence of PhHV-1 in dead (56.2%) and live pups on the IOM (57.8%) does not support this.

315 The pathogenicity of PhHV-1 is poorly understood but the significant statistical correlation
316 with hepatic necrosis, thymic atrophy and mouth ulcers found in this study may point to a
317 similar pathogenesis to that seen in harbour seals (Borst et al. 1986, Goldstein et al. 2005,
318 Himworth et al. 2010). This suggests that, even though grey seal pups have been shown to be
319 less affected by this virus than the sympatric harbour seal (Martina et al. 2002), PhHV-1 may
320 be a key component contributing to neonatal mortality both in the wild and in rehabilitation
321 facilities. The key question of what causes the transition between PhHV-1 shedding and
322 development of systemic disease in any seal species remains to be elucidated. Host factors
323 such as immunosuppression or age at initial challenge, both factors known to affect the
324 progression and outcome of FeHV1 in cats (Gaskell & Willoughby 1999), are likely to play a
325 part in PhHV-1 pathogenesis and should be investigated further.
326
327 The study also demonstrated that nasal shedding of PhHV-1 in yearling grey seals (27.8%) is
328 most likely due to stress-induced recrudescence. Post-weaning to yearling grey seals undergo
329 dramatic physiological changes including a switch in body composition from 13% to 20%
330 protein at the expense of fat, which decreases from 40% to 12% body weight (Hall &
331 McConnell 2007). A plausible hypothesis may be that there is some form of energetic trade-
332 off between resources for immunity and protein deposition, possibly compounded by the
333 stress of returning to the breeding colony, which leads to this viral reactivation. The initial
334 source of exposure of pups to PhHV-1 is unknown but extrapolation from other species and
335 other neonatal alpha herpesviruses would suggest that both perinatal transmission from
336 maternal vaginal secretions and horizontal pup to pup transmission may be involved.
337 Pregnancy associated immuno-suppression has been previously demonstrated in grey seals
338 (King et al. 1994) and the periparturient drop in immunity is a recognised trigger for
339 transmission of parasites and viruses in numerous domestic animal species (Xiao et al. 1994,

Waller et al. 2004, Cattadori et al. 2005). As a result, Goldstein et al. (2004) speculated that in harbour seals, cows may be the source of infection through periparturient recrudescence of PhHV-1 excreted in nasal and vaginal secretions. To investigate this further in grey seals, nasal swabs and vaginal swabs should be taken from adult females immediately before and after pupping and from adult females out-with the breeding season. If PhHV-1 shedding is linked to stress, monitoring nasal viral titres could be used as an indicator of welfare/stress in seals in rehabilitation centres, informing indirectly on the effects of various husbandry practices. Rehabilitation centres would also provide an accessible system in which to study PhHV-1 transmission and pathogenesis in grey seal pups as has been performed previously in harbour seals (Goldstein et al. 2004, 2005).

To help further elucidate the pathogenesis and tropism of this virus, the quantitation of virus load or transcription levels of RNA in individual tissues would be worthwhile. Similarly, localisation of the virus within lesions would be of use and given the absence of a suitable immunohistochemical method, development of an in-situ hybridisation probe to localise the pathogen would be justified.

This work focused only on a single breeding season and a single colony, therefore care should be taken in extrapolating these findings to successive seasons and other colonies. Replication of this study over several seasons or after regular intervals would determine if these findings are typical for grey seal breeding colonies. However, the high prevalence of PhHV-1in the grey seal pups suggests a widespread exposure to the virus at birth, similar to that seen with other alpha herpesviruses.

Table and figure titles

Table 1 Prevalence of PhHV-1. Number of positive animals (%: percentage of each group).

Figure 1 PhHV-1 status of stranded dead and free-ranging dead grey seal pups presenting with differing degrees of thymic atrophy; Bars represent 95% confidence interval; Significance brackets represent result of generalised linear model comparing prevalence of PhHV-1 within groups of pups presenting each degree of thymic atrophy

Supplementary tables

Table S 1 Categorical risk factors, using univariate analysis, for detecting Phocid herpesvirus 1 from nasal swabs from grey seals; (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Table S 2 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live free-ranging grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Table S 3 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live free-ranging and stranded grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Table S 4 Odds ratio of finding lesions in pups with a positive PhHV-1 PCR status. (OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Acknowledgements:

This work and JLB's PhD studentship were funded by the Moredun Research Institute and the Royal Zoological Society of Scotland. JC was supported by a Wellcome Trust Biomedical Vacation Scholarship.

We wish to thank the Sea Mammal Research Unit at the University of St Andrews for assistance with field sampling (Simon Moss, Matt Bivins, Kelly Robinson, Paula Redman, Chris McKnight and Amanda Stansbury), mapping and statistical advice (Bernie McConnell and Mike Lonergan); the Scottish SPCA National Wildlife Rescue Centre (Colin Seddon, Claire Stainfield and staff) for sample collection and coordination; Clare Underwood, Jeanie Finlayson and Val Forbes of Moredun Research Institute Pathology department for excellent histopathologcal and immunohistochemical preparations; and Dylan Turnbull and Ellie Laming of Moredun Virology department for expert molecular biology assistance. All sampling of live free-ranging animals was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures) Act, 1986. Stranded grey seal pups were sampled as part of the routine health assessment procedure.

Reference list

Baily JL (2014) The pathology and occurrence of pathogens in Scottish grey seals (Halichoerus grypus). Thesis, University of St Andrews, Scotland, UK

Borst GH, Walvoort HC, Reijnders PJ, Kamp JS van der, Osterhaus AD (1986) An outbreak of a

412 herpesvirus infection in harbor seals (Phoca vitulina). J Wildl Dis 22:1–6

413 Brukman A, Enquist LW (2006) Suppression of the interferon-mediated innate immune response by
414 pseudorabies virus. J Virol 80:6345–6356

415 Caswell JL, Williams KJ (2007) Respiratory system. In: Maxie MG (ed) Jubb Kennedy, and Palmer's
416 Pathology of Domestic Animals, 5th edn. Elsevier Limited, Philadelphia, p 523–653

417 Cattadori IM, Boag B, Bjornstad ON, Cornell SJ, Hudson PJ (2005) Peak shift and epidemiology in a
418 seasonal host-nematode system. ProcBiol Sci 272:1163–1169

419 Ehlers B, Borchers K, Grund C, Frolich K, Ludwig H, Buhk HJ (1999) Detection of new DNA
420 polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and
421 deoxyinosine-substituted primers. Virus Genes 18:211–220

422 Gaskell R, Dawson S, Radford A, Thiry E (2007) Feline herpesvirus. Vet Res 38:337–354

423 Gaskell R, Willoughby K (1999) Herpesviruses of carnivores. Vet Microbiol 69:73–88

424 Goldstein T, Mazet JA, Gulland FMD, Rowles T, Harvey JT, Allen SG, King DP, Aldridge BM, Stott
425 JL (2004) The transmission of phocine herpesvirus-1 in rehabilitating and free-ranging Pacific
426 harbor seals (Phoca vitulina) in California. Vet Microbiol 103:131–141

427 Goldstein T, Mazet JA, Lowenstine LJ, Gulland FM, Rowles TK, King DP, Aldridge BM, Stott JL
428 (2005) Tissue distribution of phocine herpesvirus-1 (PhHV-1) in infected harbour seals (Phoca
429 vitulina) from the central Californian coast and a comparison of diagnostic methods.. J Comp
430 Pathol 133:175–183

431 Gulland FM, Lowenstine LJ, Lapointe JM, Spraker T, King DP (1997) Herpesvirus infection in
432 stranded Pacific harbor seals of coastal California. J Wildl Dis 33:450–458

433 Hall AJ, McConnell BJ (2007) Measuring changes in juvenile gray seal body composition. Mar
434 Mammal Sci 23:650–665

435 Harder TC, Harder M, Vos H, Kulonen K, Kennedy-Stoskopf S, Liess B, Appel MJ, Osterhaus AD
436 (1996) Characterization of phocid herpesvirus-1 and -2 as putative alpha- and
437 gammaherpesviruses of North American and European pinnipeds. J Gen Virol 77 (Pt 1):27–35

438 Himworth CG, Haulena M, Lambourn DM, Gaydos JK, Huggins J, Calambokidis J, Ford JK,
439 Zaremba K, Raverty S (2010) Pathology and epidemiology of phocid herpesvirus-1 in wild and
440 rehabilitating harbor seals (Phoca vitulina richardsi) in the northeastern Pacific. J Wildl Dis
441 46:1046–1051

442 King DP, Lowe KA, Hay AW, Evans SW (1994) Identification, characterisation, and measurement of
443 immunoglobulin concentrations in grey (Haliocherus grypus) and common (Phoca vitulina)
444 seals. DevCompImmunol 18:433–442

445 Kovacs KM, Lavigne DM (1986) Maternal Investment and Neonatal Growth in Phocid Seals. J Anim
446 Ecol 55:1035–1051

447 Martina BE, Jensen TH, Bildt MW van de, Harder TC, Osterhaus AD (2002) Variations in the
448 severity of phocid herpesvirus type 1 infections with age in grey seals and harbour seals. Vet
449 Rec 150:572–575

450 Osterhaus AD, Yang H, Spijkers HE, Groen J, Teppema JS, Steenis G van (1985) The isolation and
451 partial characterization of a highly pathogenic herpesvirus from the harbor seal (Phoca vitulina).
452 Arch Virol 86:239–251

453 Roth SJ, Tischer BK, Kovacs KM, Lydersen C, Osterrieder N, Tryland M (2013) Phocine herpesvirus
454 1 (PhHV-1) in harbor seals from Svalbard, Norway. Vet Microbiol 164:286–292

455 Schlafer DH, Miller RB (2007) Female genital system. In: Maxie MG (ed) Jubb Kennedy, and
456 Palmer's Pathology of Domestic Animals, 5th edn. Elsevier Limited, Philadelphia, p 429–564

457 Snyder PW (2007) Diseases of immunity. In: McGavin MD, Zachary JF (eds) Pathologic basis of
458 veterinary disease, 4th edn. Mosby Elsevier, St. Louis, p 193–252

459 Team RC (2013) R: A language and environment for statistical computing. (V R Foundation for
460 Statistical Computing Austria, Ed.). http://wwwR-project.org/

461 Thonur L, Maley M, Gilray J, Crook T, Laming E, Turnbull D, Nath M, Willoughby K (2012) One-
462 step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine
463 herpesvirus 1 and bovine parainfluenza virus 3. BMC Vet Res 8:37

464 Walle GR Van de, Jarosinski KW, Osterrieder N (2008) Alphaherpesviruses and chemokines: pas de
465 deux not yet brought to perfection. J Virol 82:6090–6097

466 Waller PJ, Rudby-Martin L, Ljungstrom BL, Rydzik A (2004) The epidemiology of abomasal
467 nematodes of sheep in Sweden, with particular reference to over-winter survival strategies. Vet
468 Parasitol 122:207–220

469 Winkler MT, Doster A, Jones C (1999) Bovine herpesvirus 1 can infect CD4(+) T lymphocytes and
470 induce programmed cell death during acute infection of cattle. J Virol 73:8657–8668

471 Xiao L, Herd RP, McClure KE (1994) Periparturient rise in the excretion of Giardia sp. cysts and
472 Cryptosporidium parvum oocysts as a source of infection for lambs. J Parasitol 80:55–59

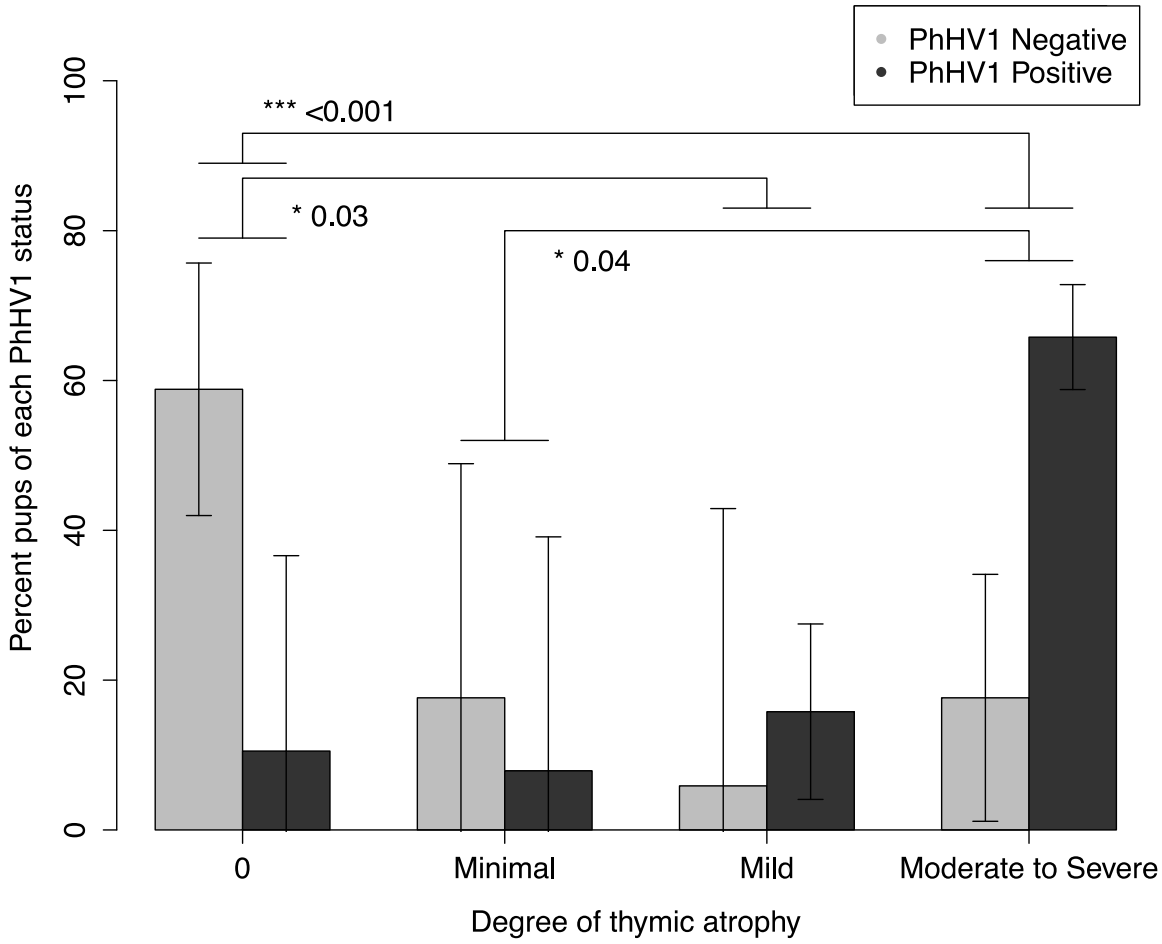
473

474 Table 1 Prevalence of PhHV-1. Number of positive animals (%: percentage of each group).

Pathogen	Stranded Live	Stranded Dead	Colony Live	Colony Dead	Colony Yearlings
PhHV-1 Nasal swab	18/29 (62%)	8/9 (89%)	52/90 (58%)	27/48 (56%)	5/18 (28%)
PhHV-1 Pooled tissue samples		6/9 (67%)		26/48 (54%)	

475

476 Figure 1:



477

478

479

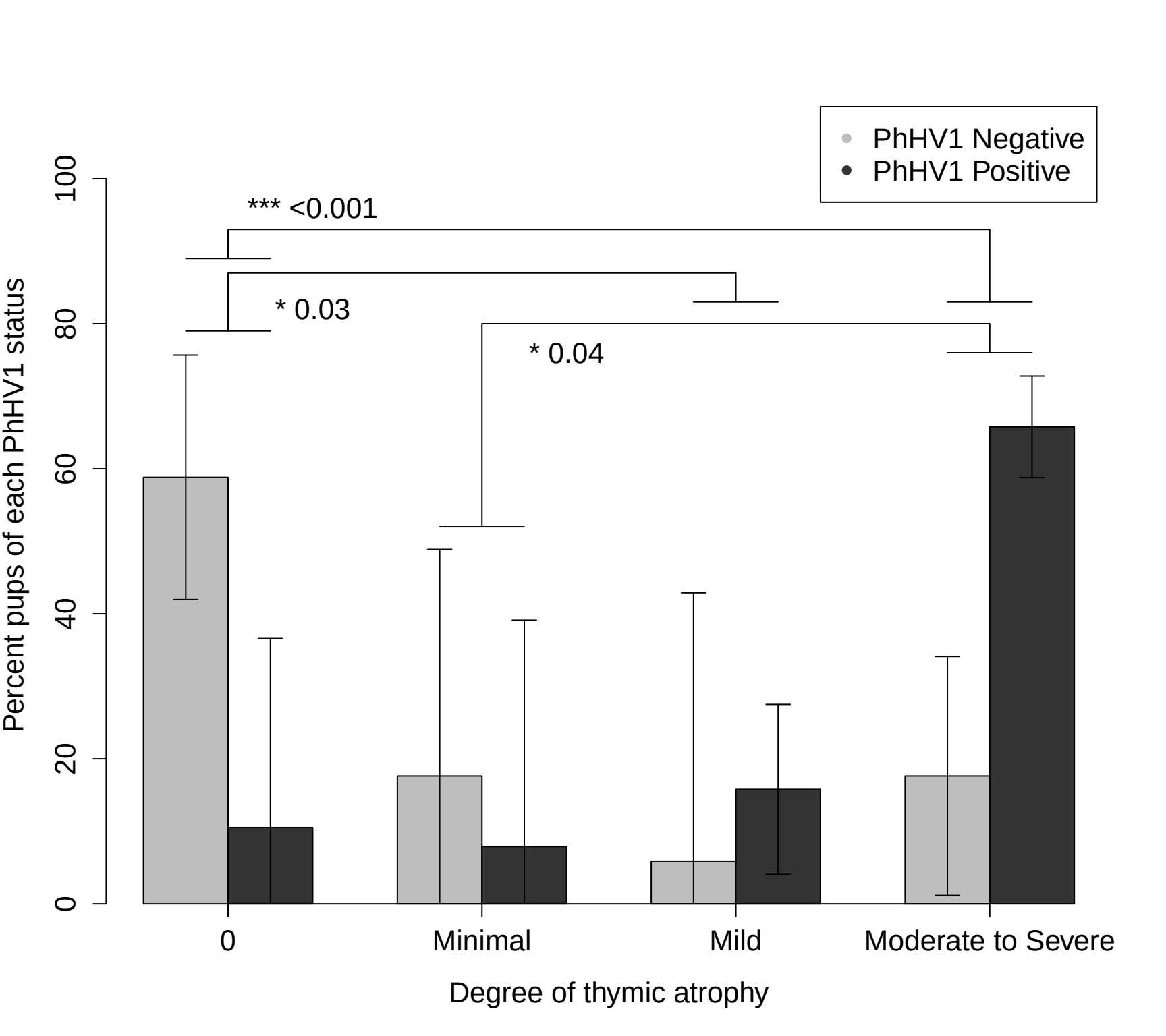

PhHV1 Negative
PhHV1 Positive
*** <0.001
* 0.03
* 0.04
Percent pups of each PhHV1 status
0
20
40
60
80
100
0
Minimal
Mild
Moderate to Severe
Degree of thymic atrophy

Table S 1 Categorical risk factors, using univariate analysis, for detecting Phocid herpesvirus 1 from nasal swabs of grey seals; (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

	Risk factor	Group	(n=)	number (%) positive	OR	95% CI	P-value	Sign.
All seals	Group	Free-ranging - live	90	52 (57.8%)	1.00			
		Free-ranging - Dead	50	27 (56.2%)	0.94	0.46, 1.91	0.863	NS
		Stranded - Live	31	18 (62.1%)	1.20	0.51, 2.82	0.683	NS
		Stranded – died/euthanised	9	8 (88.9%)	5.85	0.7,48.73	0.103	NS
		Live free-ranging yearlings	19	5 (27.8%)	0.28	0.06 0.86	0.025	*
Free-ranging live and dead pups	Live/dead	Dead	50	27 (56.2%)	1.00			
		Live	90	52 (57.8%)	1.06	0.52, 2.16	0.863	NS
	Sex	Female	65	34 (52.3%)	1			
		Male	72	45 (62.5%)	1.52	0.77, 3	0.228	NS
	Mass range (kg)	<12	13	5 (38.5%)	1			
		12-25	73	31 (42.5%)	1.18	0.35, 3.96	0.787	NS
		25-31	13	11 (84.6%)	8.8	1.35, 57.43	0.02	*
		>31	39	39 (28.3%)	7.31	1.83, 29.2	0.004	**
Free-ranging Live pups only	Time point	Early	30	11 (36.7%)	1.00			
		Mid	30	20 (66.7%)	3.45	1.19, 9.99	0.022	*
		Late	30	21 (70%)	4.00	1.37, 11.84	0.011	*
	Sampling site	Tidal boulder beach	30	22 (73.3%)	1.00			
		Rocky pools	30	18 (60%)	0.55	0.18, 1.62	0.276	NS
		Muddy grassy slope	30	12 (40%)	0.24	0.08, 0.72	0.011	*
	Coat	Stage II	41	16 (39%)	1.00			
		Stage III	13	9 (69.2%)	3.52	0.93, 13.35	0.065	NS
		Stage IV - Moult	27	21 (77.8%)	5.47	1.81, 16.48	0.003	**
		Stage V- Adult coat	9	6 (66.7%)	3.12	0.68, 14.31	0.142	NS

Table S 2 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live free-ranging grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Variable		Coefficient.	S.E. of coef.	Odds ratio (OR)	95% CI of OR	p-value
Intercept		-2.89	0.92	-	-	-
Mass	Mass in kg	0.17	0.017	1.15	1.08,1.22	<0.001
Site	Tidal boulder beach	-	-	1	-	-
	Rocky pools	-1.75	0.72	0.55	0.18, 1.62	0.015
	Muddy grassy slope	-2.22	0.71	0.24	0.08, 0.72	0.002

Table S 3 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live free-ranging and stranded grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Variable		Coefficient	S.E.of coef.	Odds ratio (OR)	95% CI of OR	p-value
Intercept		-3.107	0.797			
Mass	Mass in kg	0.128	0.03	1.09	1.04,1.14	<0.001
Colony	Free-ranging pups	-	-	1	-	-
	Stranded pups	1.592	0.55	1.2	0.51, 2.82	0.003

Table S 4 Odds ratio of finding lesions in pups with a positive PhHV-1 PCR status. (OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001).

Lesion	PhHV-1 status	number (%) positive	OR	95% CI	P-value	Sign.
Adrenal necrosis	HV - HV +	1 (5.9%) 7 (16.7%)	3.20	0.36, 28.23	0.29	NS
Hepatic necrosis	HV - HV +	1 (5.9%) 20 (50%)	16.00	1.93, 132.39	0.01	*
Multifocal hepatitis	HV - HV +	4 (23.5%) 16 (38.1%)	2.00	0.55, 7.21	0.289	NS
Thymic Atrophy	HV - HV +	4 (23.5%) 31 (81.6%)	14.39	3.59, 57.71	<0.001	***
Buccal ulceration	HV - HV +	1 (5.9%) 17 (40.5%)	10.88	1.32, 89.93	0.027	*
Tongue ulceration	HV - HV +	3 (17.6%) 11 (26.2%)	1.66	0.4, 6.88	0.488	NS
Keratitis	HV - HV +	0 (0%) 4 (9.5%)	33091159	0, Inf	0.995	NS
Uveitis	HV - HV +	0 (0%) 5 (12.5%)	44909430	0, Inf	0.995	NS
Interstitial pneumonia	HV - HV +	7 (41.2%) 22 (52.4%)	1.57	0.5,4.91	0.437	NS