

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

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4 **Running page head: PhHV1 in grey seals**

5

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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 herpesvirus 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

51 **Introduction**

52 Phocid herpesvirus-1 (PhHV-1) is an alphaherpesvirus which infects both grey (*Halichoerus*
53 *grypus*) and harbour (*Phoca vitulina*) seals in the Eastern Atlantic and Pacific Oceans (Borst
54 et al. 1986, Harder et al. 1996, Gulland et al. 1997, Martina et al. 2002, Himworth et al.
55 2010). The alphaherpesvirus subfamily consists of large double-stranded DNA viruses of
56 variably narrow to broad host range, typically characterised by rapid replication in cell
57 culture, lytic infection and the ability to develop latent infections in sites such as the
58 trigeminal ganglia (Caswell & Williams 2007). Alphaherpesviruses are responsible for a
59 number of economically important diseases in domestic species including Bovine herpesvirus
60 1 (BoHV-1) which causes infectious bovine rhinotracheitis in calves and abortion in cows,
61 Suid herpesvirus which causes Aujeszky's disease and abortion in pigs and Equid
62 herpesviruses 1 and 4 which result in respiratory and neurological diseases in horses. Another
63 feature of many alphaherpesviruses is the ability to induce immunosuppression (Winkler et
64 al. 1999, Brukman & Enquist 2006, Van de Walle et al. 2008).

65

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

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

81

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

88

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

96

97

98 **Materials and methods**

99 **Animals and sampling**

100 Grey seal pups and yearlings were sampled during the pupping season of autumn 2011. Free-
101 ranging, live (n=90) and dead (n=50) grey seal pups and live yearlings (n=19) were sampled
102 on the Isle of May (IOM), Firth of Forth, UK. Live grey seal pups were sampled from three
103 distinct sites on the Isle of May which had different substrate and animal movement
104 characteristics (tidal boulder beach with twice daily congregations of pups due to
105 displacement by the tide (n=30); muddy/grassy slope with sedentary pups (n=30) and
106 stagnant rocky pools with sedentary pups (n=30)) and at three different time points (early,
107 mid and late pupping season). Dead grey seal pups were collected opportunistically
108 throughout the season from varied sites on the colony and within 48h of death. Additionally,
109 live-stranded grey seal pups (n=31) were sampled within 24h of arrival at the Scottish
110 Society for the Prevention of Cruelty to Animals National Wildlife Centre (SSPCA), Fife as
111 part of the routine health assessment procedure to determine suitability for care and
112 rehabilitation prior to release back into the wild. All sampling of live animals on the Isle of
113 May was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as
114 issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures) Act,
115 1986. All sampling of live animals submitted to the rehabilitation centre was for diagnostic
116 purposes to determine the cause of stranding and future treatment regime.

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

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

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

125

126 A nasal swab was obtained from all live and dead animals examined, using a nylon flocculated
127 swab and placed into universal transport medium (UTM) (swab and UTM: Catalogue no.
128 346C, Sterilin, Newport, UK). Swabs were initially stored at 4°C and subsequently frozen at -
129 80°C within 12 hours of collection until analysis. A full post-mortem examination was
130 performed on all dead pups and samples collected for histopathology. Pooled representative
131 samples of 10 organs (liver, spleen, mesenteric lymph node, ileo-caeco-colic junction,
132 kidney, left cranial lung lobe, bronchial lymph node, right ventricle, tonsil and brain) were
133 collected aseptically, placed in a sterile gentleMACS™ M tube (Miltenyi Biotec, Bisley, UK)
134 and frozen at -80°C until analysis.

135

136 **Sample processing and analysis**

137 Nasal swabs in universal transport medium were placed into a sonicator bath for 30s and then
138 centrifuged at 2000g for 10min at 4°C. The resultant supernatant was stored at -80°C until
139 analysis. Pooled tissue samples were homogenised with 5ml viral transport medium using a
140 Dispomix homogeniser (Miltenyi Biotec Ltd., Bisley, UK), centrifuged at 2000g for 10min at
141 4°C and the resultant supernatant stored at -80°C until analysis. Nucleic acids were extracted
142 from supernatants using the NorDiagViral NA Arrow automated extraction robot (Alere,
143 Stockport, UK) as per manufacturer's instructions and stored at -20°C. Extraction controls
144 (water or PBS) were processed with every batch of tissue and swab samples.

145

146 **Detection of PhHV-1 specific nucleic acids**

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

165

166 **Histopathology and immunohistochemistry**

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

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

176

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

192

193 **Statistical analyses**

194 Prevalence data were analysed using the R statistical software package (R Core Team 2013).

195 To investigate significant differences in prevalence of PhHV-1 in nasal swabs between

196 groups Fisher's exact tests were performed. For univariate analysis generalized linear models
197 (GLMs) with a binomial distribution family and a logit link function was used to evaluate the
198 effects of different morphologic measurements (standard length, body mass, axillary girth),
199 age (stage of pup development as a proxy), sex, sampling time, colony (stranded versus free-
200 ranging animals) and ground substrate on the presence or absence of PhHV-1. Multivariate
201 logistic regression was performed using R with a forward stepping algorithm and a p value of
202 ≤ 0.05 for inclusion in the model based on the likelihood ratio test. Any stranded grey seal
203 pups which died at the rehabilitation centre were excluded from the multivariate analysis of
204 PhHV-1 nasal swab status to avoid repeat sampling of the individual animals swabbed on
205 entry to the rehabilitation centre. As length, body mass, girth and pup stage were highly
206 correlated with each other, and so as not to violate the assumption in generalised linear
207 models concerning the independence of independent variables, body mass was chosen as the
208 most reliable, independently verified and reproducible of the morphometric measurements in
209 the present study and retained for further analyses. Akaike's Information Criterion (AIC) was
210 used to compare models and choose the most parsimonious for each variable. Residual
211 analysis was used to assess goodness of fit.

212

213 **Results**

214 **Prevalence of PhHV-1 in grey seal pups and yearlings**

215 The prevalence of PhHV-1 in nasal swabs of all pups was 59.6% (105/176) with a prevalence
216 of 62% (18/29) in stranded, live pups presented for rehabilitation, 57.8% (52/90) in free-
217 ranging live pups on the IOM colony and 56.3% (27/48) dead pups on the IOM colony. The
218 group with the highest prevalence was the stranded dead pup group with a prevalence of 89%
219 (8/9, Table 1). Yearlings had a significantly lower prevalence of PhHV-1 (28%) than pups
220 (60%) ($p=0.008$; Fisher's exact test).

221

222 Odds ratios for categorical risk factors were determined for PhHV-1 (Table S 1). Live and
223 dead free-ranging pups sampled in the late pupping season had significantly higher odds of
224 being PhHV-1 positive in nasal swabs, as determined by presence of PhHV-1 nucleic acids,
225 than pups sampled in the early pupping season ($p < 0.001$, GLM). Also, free-ranging pups
226 sampled at stage I (youngest age sampled) were significantly less likely to be shedding
227 PhHV-1 in nasal secretions compared to pups in any of stages III, IV and V ($p = 0.004$;
228 $p = 0.002$; $p = 0.024$, respectively).

229

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

239

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

246

247 **Tissue samples**

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

252

253 **Pathology**

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

265

266

267 **Discussion**

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

280

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

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

293

294 Given the non-invasive nature of the study, it was not possible to assess tissue presence of
295 PhHV-1 in live pups. However, the number of pups infected with PhHV-1 is likely to be
296 substantially higher than the number of pups shedding the virus in nasal secretions. As a
297 crude measure, if one assumes that stage II and stage V pups are of sufficiently different ages
298 (average 4 days vs 18 days respectively) and that the duration of PhHV-1 nasal shedding does
299 not exceed 14 days (7-19 days according to Goldstein et al. (2004)), shedding in stage V pups
300 is likely to represent newly infected pups. The combined prevalence of PhHV-1 in stage II
301 (39%) and stage V pups (66.7%) found in this study would support a very high risk of
302 exposure of pups to the virus.

303

304 The significantly higher prevalence of PhHV-1 among live, free-ranging pups on the tidal
305 boulder beach site compared to the other two sites may be a consequence of regular
306 displacement and subsequent crowding of these pups due to the twice daily high tides. This
307 may lead to higher stress levels and also increased contact between pups and other adult
308 seals. The very high prevalence of PhHV-1 found in pups dying at the rehabilitation centre
309 (88.9%) may result from stress-induced viral replication and/or horizontal transmission of
310 virus within individuals at the rehabilitation centre, such as that described previously in
311 harbour seals (Goldstein et al. 2004, Himworth et al. 2010). The role of stress in the spread
312 and pathogenesis of PhHV-1 warrants further investigation as the comparable prevalence of
313 PhHV-1 in dead (56.2%) and live pups on the IOM (57.8%) does not support this.

314

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,

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

350

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

356

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

363

364 **Table and figure titles**

365

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

367

368 Figure 1 PhHV-1 status of stranded dead and free-ranging dead grey seal pups presenting

369 with differing degrees of thymic atrophy; Bars represent 95% confidence interval;

370 Significance brackets represent result of generalised linear model comparing prevalence of

371 PhHV-1 within groups of pups presenting each degree of thymic atrophy

372

373 **Supplementary tables**

374

375 Table S 1 Categorical risk factors, using univariate analysis, for detecting Phocid herpesvirus

376 1 from nasal swabs from grey seals; (n=: group size; OR: odds ratio; 95% CI: 95%

377 confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant;

378 *: p<0.05; **: p<0.01; ***: p<0.001).

379

380 Table S 2 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live

381 free-ranging grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95%

382 CI: 95% confidence interval.

383

384 Table S 3 Multivariate logistic regression analysis for PhHV-1 PCR of nasal swabs in live

385 free-ranging and stranded grey seal pups. S.E. of coef: Standard error of coefficient; OR:

386 odds ratio; 95% CI: 95% confidence interval.

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

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403 sampling of live free-ranging animals was carried out under UK Home Office Project (No.
404 60/4009) and Personal Licences as issued to the Sea Mammal Research Unit under the
405 Animals (Scientific Procedures) Act, 1986. Stranded grey seal pups were sampled as part of
406 the routine health assessment procedure.

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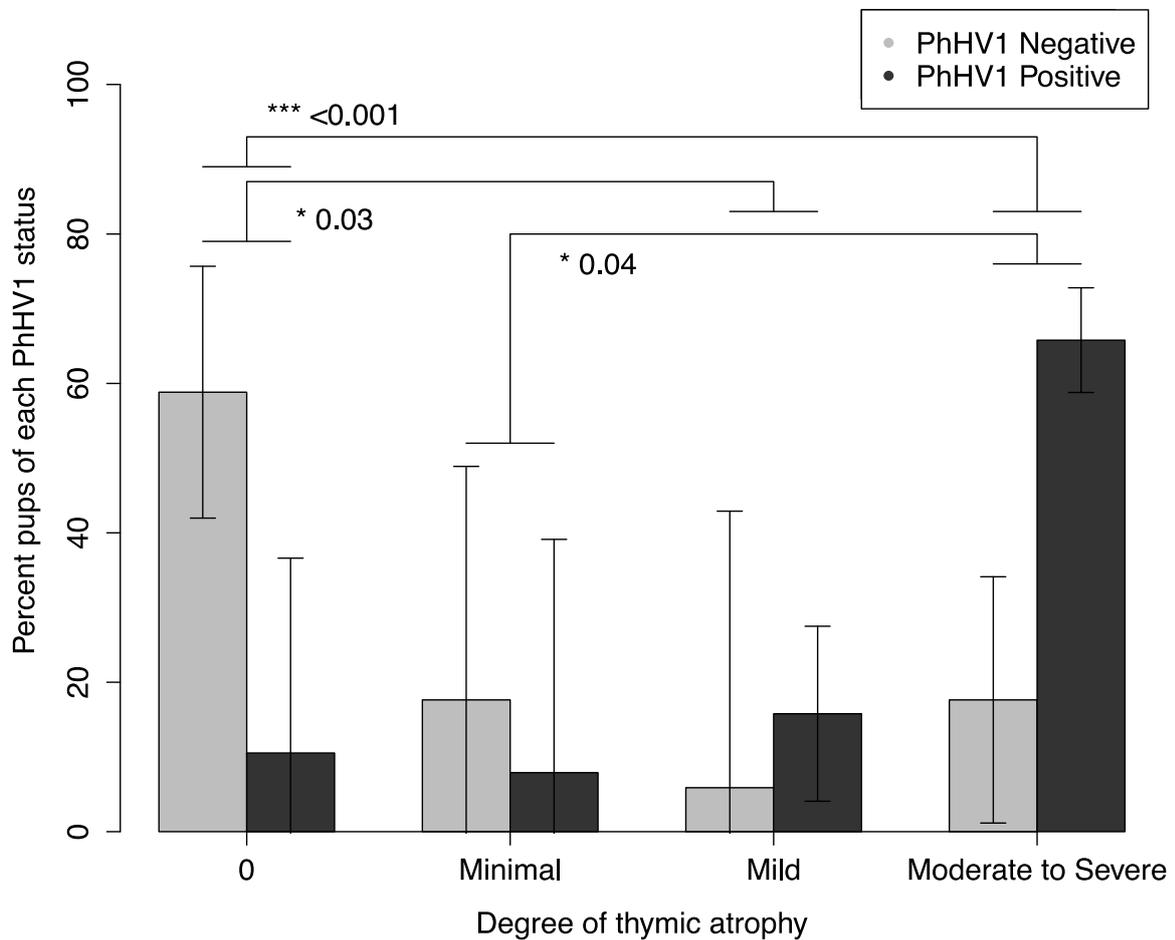
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474 Table 1 Prevalence of PhHV-1. Number of positive animals (%: percentage of each group).

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%)	

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476 Figure 1:



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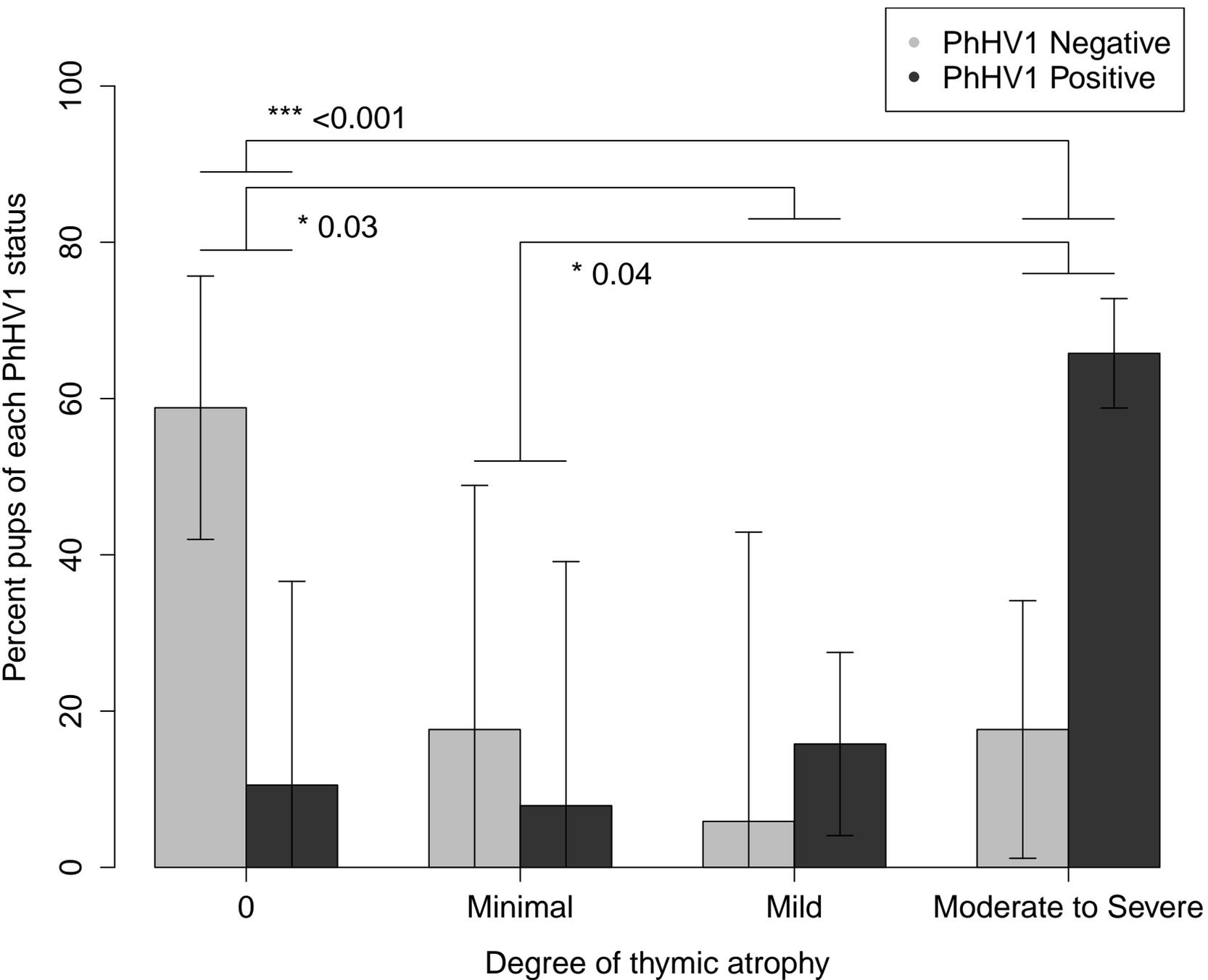


Table S 1 Categorical risk factors, using univariate analysis, for detecting Phocid herpesvirus 1 from nasal swabs of grey seals; (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: 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 - Moulting	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 -	1 (5.9%)	3.20	0.36, 28.23	0.29	NS
	HV +	7 (16.7%)				
Hepatic necrosis	HV -	1 (5.9%)	16.00	1.93, 132.39	0.01	*
	HV +	20 (50%)				
Multifocal hepatitis	HV -	4 (23.5%)	2.00	0.55, 7.21	0.289	NS
	HV +	16 (38.1%)				
Thymic Atrophy	HV -	4 (23.5%)	14.39	3.59, 57.71	<0.001	***
	HV +	31 (81.6%)				
Buccal ulceration	HV -	1 (5.9%)	10.88	1.32, 89.93	0.027	*
	HV +	17 (40.5%)				
Tongue ulceration	HV -	3 (17.6%)	1.66	0.4, 6.88	0.488	NS
	HV +	11 (26.2%)				
Keratitis	HV -	0 (0%)	33091159	0, Inf	0.995	NS
	HV +	4 (9.5%)				
Uveitis	HV -	0 (0%)	44909430	0, Inf	0.995	NS
	HV +	5 (12.5%)				
Interstitial pneumonia	HV -	7 (41.2%)	1.57	0.5,4.91	0.437	NS
	HV +	22 (52.4%)				