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Brain transcriptomes of harbor seals demonstrate gene expression patterns of animals undergoing a metabolic disease and a viral infection

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Diseases of marine mammals can be difficult to diagnose because of the life history and protected status of these animals. Stranded marine mammals have been a particularly useful resource to discover and comprehend the diseases that plague these top predators. Additionally, advancements in high-throughput sequencing (HTS) has contributed to the discovery of novel pathogens in these animals. In this study, we use a combination of HTS and stranded harbor seals (*Phoca vitulina*) to better understand a known and unknown brain disease. To do this, we used transcriptomics to evaluate brain tissues from seven neonatal harbor seals that expired from an unknown cause of death (UCD) and compared them to four neonatal harbor seals that had confirmed phocine herpesvirus (PhV-1) infections in the brain. Comparing these two disease states we found that UCD animals showed a significant abundance of fatty acid metabolic transcripts in their brain tissue, thus we speculate that a fatty acid metabolic dysregulation contributed to the death of these animals. Furthermore, we were able to describe the response of four young harbor seals with PhV-1 infections in the brain. PhV-1 infected animals showed a significant ability to mount an innate and adaptive immune response, especially to combat viral infections. Our data also suggests that PhV-1 can hijack host pathways for DNA packaging and exocytosis. This is the first study to use transcriptomics in marine mammals to understand host and viral interactions and assess the death of stranded marine mammals with an unknown disease. Furthermore, we show the value of applying transcriptomics on stranded marine mammals for disease characterization.

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12

13 **Abstract**

14 Diseases of marine mammals can be difficult to diagnose because of the life history and
15 protected status of these animals. Stranded marine mammals have been a particularly useful
16 resource to discover and comprehend the diseases that plague these top predators. Additionally,
17 advancements in high-throughput sequencing (HTS) has contributed to the discovery of novel
18 pathogens in these animals. In this study, we use a combination of HTS and stranded harbor seals
19 (*Phoca vitulina*) to better understand a known and unknown brain disease. To do this, we used
20 transcriptomics to evaluate brain tissues from seven neonatal harbor seals that expired from an
21 unknown cause of death (UCD) and compared them to four neonatal harbor seals that had
22 confirmed phocine herpesvirus (PhV-1) infections in the brain. Comparing these two disease
23 states we found that UCD animals showed a significant abundance of fatty acid metabolic
24 transcripts in their brain tissue, thus we speculate that a fatty acid metabolic dysregulation

25 contributed to the death of these animals. Furthermore, we were able to describe the response of
26 four young harbor seals with PhV-1 infections in the brain. PhV-1 infected animals showed a
27 significant ability to mount an innate and adaptive immune response, especially to combat viral
28 infections. Our data also suggests that PhV-1 can hijack host pathways for DNA packaging and
29 exocytosis. This is the first study to use transcriptomics in marine mammals to understand host
30 and viral interactions and assess the death of stranded marine mammals with an unknown
31 disease. Furthermore, we show the value of applying transcriptomics on stranded marine
32 mammals for disease characterization.

33

34 **Introduction:**

35

36 *The combination of high-throughput sequencing (HTS) and stranded marine mammals for*

37 *disease discovery*

38 The health of wild marine mammal populations is difficult to assess because of their
39 unknown population sizes, large distributions, and protected status. Stranded or vulnerable
40 animals found ashore, have been essential for scientists to identify causes of marine mammal
41 deaths. For example, important pathogens like phocine distemper virus (PDV), and *Leptospira*,
42 were originally discovered in stranded marine mammals (Vedros et al., 1971; Osterhaus et al.,
43 1988). Yet, a large majority of marine mammal deaths remain unknown. In 2007, it was reported
44 that only 56% of marine mammal mortality events had a known cause of death (Gulland & Hall,
45 2007), leaving the pathogens and physiological causes of many diseases to be discovered.
46 However, the introduction of high-throughput sequencing (HTS) has led to the identification of
47 many more marine mammal pathogens, such as seal and California sea lion anellovirus, phocine
48 herpesvirus 7, and seal parvovirus (Ng et al., 2009, 2011; Bodewes et al., 2013; Kuiken et al.,

49 2015). Therefore, the combination of stranded animals and HTS are vital resources for the
50 discovery of marine mammal diseases.

51 *HTS and gene expression studies to understand disease in marine mammals*

52 Although the discovery and characterization of new disease agents can aid in the
53 conservation of marine mammal populations, there are many described marine mammal diseases
54 that are not fully understood. For example, phocine herpesvirus-1 (PhV-1) was discovered in
55 1985 and is highly abundant in North American harbor seal adults (99%). It is particularly
56 pathogenic to young seals causing ~46% mortality (Osterhaus et al., 1985; Harder et al., 1996;
57 Gulland et al., 1997). Despite PhV-1's deleterious impacts, we have little understanding of the
58 effects of PhV-1 on host gene expression. Previous marine mammal studies have identified
59 pinniped immune responses against PhV-1 using enzyme-linked immunosorbent (ELISA), but
60 this offers minimal information about the disease (Harder et al., 1998). In addition, marine
61 mammal studies have applied targeted gene expression techniques using RT-qPCR to understand
62 immune and endocrine responses to immunotoxins and physiological changes (Neale et al.,
63 2005; Hammond, 2005; Tabuchi et al., 2006). However, ELISA and RT-qPCR target a limited
64 number of host gene expressions; thus they do not represent the global host response.

65 HTS is a powerful resource for assessment of both the etiology of a disease and the
66 response of the host during disease events. For example, using transcriptomic analysis, scientists
67 were able to determine that a toxin caused a mass mortality event of abalone, pinpoint the origin
68 of the toxin, and access the genetic effects on the abalone population (De Wit et al., 2014).
69 However, transcriptomic analysis has rarely been used to comprehend the effects of stressors and
70 diseases on marine mammal health. There has been some increase in marine mammal
71 transcriptomic studies, such as a study by Hoffman *et al.*, which suggests that *post-mortem*

72 samples can be reliable resources for genomic studies (Hoffman et al., 2013). Yet, there have
73 been few studied that use transcriptomics to measure physiological stress responses in marine
74 mammals, and there have been no studies that looked at pathogen responses in these megafauna
75 (Mancia et al., 2014; Niimi et al., 2014; Khudyakov et al., 2015a,b; Fabrizius et al., 2016).

76 *Former work on a harbor seal stranding event using HTS*

77 In our previous work, we used harbor seals and meta-transcriptomics to identify potential
78 neurotropic bacteria and viruses in live stranded harbor seals that later died in a rehabilitation
79 center (Rosales & Vega Thurber, 2015). Due to the unknown etiology of the stranding, we
80 termed these animals involved as seals with an “unknown cause of death” or “UCD.” These
81 seven animals had neuronal necrosis in the cortex and cerebellum, which veterinarians
82 hypothesized was due to hypoxia, exposure to toxins, poor nutrition, or a viral infection.
83 However, a viral etiology was thought to be the most likely culprit given the distribution and
84 characteristics of the brain lesions, and general gross pathology of the animals.

85 Additionally, we used PhV-1 infected harbor seals, to benchmark our methods and to
86 compare our analysis to animals with a described disease. Our analysis showed no evidence of a
87 viral infection in UCD samples, but we were able to detect PhV-1 in PhV-1 infected samples.
88 Interestingly, in our study, we found a significant presence of *Burkholderia* bacteria in UCD
89 animals. Yet, necropsy reports were contradictory to this finding suggesting that either
90 *Burkholderia* were part of a secondary or opportunistic infection or elusive in the original
91 dissections (Rosales & Vega Thurber, 2015). Therefore, this leaves the cause of death of this
92 cohort of UCD animals unresolved.

93 In this study, we further evaluated this dataset by using transcriptomic analysis to better
94 understand the cause of death of the seven neonatal harbor seals that died from an unknown brain

95 disease. Additionally, we aimed to use transcriptomics to characterize the gene expression of
96 four neonatal harbor seals with a known PhV-1 brain infection. We hypothesized that animals
97 responding to a PhV-1 infection should exhibit increases in host-virus response genes, while
98 UCD animals would have characteristic gene repertoires of animals with bacterial and/or
99 hypoxia, exposure to toxins, or poor nutrition. To examine this, we looked at significant gene
100 expression alterations of UCD and PhV-1 infected harbor seals.

101

102 **Materials and Methods:**

103 This work was authorized by the National Marine Fisheries (NMFS) for possession of
104 tissue samples from stranded marine mammals. This work is in compliance with the Marine
105 Mammal Protection Act (MMPA) regulation 50 CR 216.22 and 216.37.

106 In this study, we aimed to use transcriptomics to identify the cause of death of harbor
107 seals that died from an unidentified brain disease and to characterize host pathways of harbor
108 seals during a PhV-1 infection. For transcriptome analysis, 11 harbor seal brain tissue samples
109 were evaluated. These seal brain tissues were kindly provided by the Marine Mammal Center
110 (MMC) in Sausalito, CA, USA, where the animals expired. Brain tissues were stored at -80°C
111 and belonged to the cerebrum with the exception of sample UCD2, which was tissue from the
112 cerebellum. Stranded animals were collected from 2009–2012, and necropsied from fresh
113 carcasses soon after death (Table 1 Date of necropsy). Samples ranged in age at the time of
114 stranding, from weaner (<1 month, n = 8) to pup (< 1 year, n = 3). All UCD harbor seals had
115 neuronal necrosis in the cortex and cerebellum. Other common disease signs described in these
116 animals were: hepatic lipidosis (4/7), spleen hemosiderosis (5/7), and spleen extramedullary
117 hematopoiesis (6/7). Table 1 details a summary of necropsy reports.

118 *Transcriptome library preparation*

119 Transcriptome libraries were prepared as previously published (Rosales & Vega Thurber,
120 2015). Briefly, a disposable pestle was used to homogenize ~0.5ng of the frozen brain sample in
121 Trizol (Life Technologies, CA). The homogenate was centrifuged for 10min at 12,000 x g at
122 4°C, and the supernatant transferred to a clean tube. For every 1 mL of Trizol, 0.2mL of
123 chloroform was added to the supernatant, vortexed briefly, and centrifuged at 10,000 x g for 18
124 minutes at 4°C. The aqueous layer was then transferred to a clean tube and equal volumes of
125 100% ethanol were added to samples, and loaded onto an RNeasy column for extraction as
126 recommended by the manufacturer (Qiagen, CA). To remove DNA, samples were exposed to 2U
127 of Turbo DNase (Life Technologies, CA) for 9 hours at 37°C. Harbor seal rRNA was removed
128 using the Ribo-Zero Kit Gold (Human-Mouse-Rat) from Epicentre (WI, USA) following the
129 manufacturer's directions. High-quality RNA was converted to cDNA using superscript II
130 Reverse Transcriptase (Life Technologies, CA). Libraries were prepared for each of the 11
131 samples using the TruSeq paired-end cluster kit v.3 from Illumina (San Diego, CA). Libraries
132 were sequenced on two lanes of the Illumina Hi-Seq 2000 platform. Each lane had a random
133 mixture of both harbor seal groups (UCD and PhV-1 infected animals).

134 *Bioinformatic quality control and analysis*

135 Using FqTrim the data was quality filtered with a minimum Phred score of 30. Sequences
136 were trimmed and adapters and poly-A tails removed (Geo Perteau, 2015). In addition, FqTrim,
137 sequences were trimmed a second time, to ensure all sequence lengths were a minimum of 75
138 bps long. Transcriptome assembly was then conducted using a combination of transcriptome-
139 guided and *de novo* methods. All quality assured sequence reads from both libraries were
140 combined and aligned to the hypothetical Weddell seal, *Leptonychotes weddellii*, transcriptome

141 (NCBI accession: PRJNA232772) , using the program Bowtie2-2.2.3 (Langmead & Salzberg,
142 2012). Aligned sequences were then used to build a *de novo* harbor seal transcriptome using
143 Trinity 2.0.6 with parameters --single, and --full-cleanup (Haas et al., 2013). Statistics for the
144 assembly were obtained with Transrate v1.0.3 (Smith-Unna et al., 2016). The longest
145 representative transcript for each component or subcomponent in the transcriptome assembly
146 was selected using trinity_reps.pl (<https://goo.gl/EGq7I6>). To calculate the number of transcripts
147 for each library, the 11 libraries were first individually aligned against the *de novo* transcriptome
148 using trinity's align_and_estimate_abundance.pl with options --aln_method bowtie2 and --
149 trinity_mode --prep_reference (Haas et al., 2013). The aligned sequences from each library were
150 counted using the script SamFilter_by_components.pl (<http://goo.gl/kkvqdK>).

151 *Differential expressed genes between UCDs and PhV-1 infected seals*

152 To normalize gene counts and determine differentially expressed genes (DEGs) between
153 animals infected with PhV-1 and those with an unknown cause of death (UCD), the count data
154 were analyzed using R version 3.2.2 with software packages Bioconductor 3.1 and DESeq2-
155 1.8.2. For each gene, DESeq2 fits a log generalized linear model with a negative binomial
156 distribution to normalize genes abundances (Love, Huber & Anders, 2014). Transcripts were
157 significantly different if they had at least an adjusted p-value ≤ 0.05 . Importantly, since in this
158 study we compared two diseases with no true control samples, a positive log fold change was
159 considered up-regulated in UCD samples, while if the log fold change was negative it was
160 considered up-regulated in PhV-1 infected samples.

161 *Gene Ontology (GO) enrichment analysis*

162 The transcriptome assembly was then annotated with an e-value of $\leq 10^{-20}$ using
163 GenesFromLocalDB.pl (goo.gl/4Zbbt5), a script that utilizes BLASTx to assign gene names to

164 transcripts using the UniProt database downloaded in 2014 (Magrane & Consortium, 2011).
165 Gene ontology (GO) was assigned to the annotated transcriptome with the script
166 GOFromGeneAnnotation.pl (<http://goo.gl/jJ4vg9>). Transcript IDs with assigned GO terms were
167 then combined with their respective DESeq p-values.

168 The software package ErmineJ 3.0.2 was then applied to evaluate the biological pathways
169 associated with each differentially expressed GO term (Lee et al., 2005). The analysis was run
170 with the options: gene score resampling (GSR, which does not require a threshold and thus
171 evaluates all p-values), a maximum gene set of 100, a minimum gene set of 20, a maximum
172 iteration of 200,000, and full resampling. GO terms with GO p-values ≤ 0.05 and a
173 multifunctionality of ≤ 0.85 were semantically summarized and visualized with REViGO with an
174 allowed similarity of 0.90, the most conservative setting (Supek et al., 2011).

175 *KEGG Analysis*

176 For KeggArray analysis, the harbor seal transcriptome was translated to protein reads with
177 TransDecoder 2.0 (Haas et al., 2013). The program KAAS (KEGG Automatic Annotation
178 Server) was used to annotate translated transcripts with BLASTx against a manually curated
179 KEGG GENES database (Kanehisa, 2000; Moriya et al., 2007). The KAAS options used were
180 ‘partial genome’ and ‘bi-directional best hit’ (BBH). KEGG ontology (KO) assignments with a
181 respective DESeq padj value ≤ 0.05 were used for further analysis. KeggArray was then utilized
182 to map KO pathways and CytoKegg, (a Cytoscape application
183 (<http://apps.cytoscape.org/apps/cytokegg>)) to visualize specific KO pathways.

184 *Spearman correlation analysis*

185 To evaluate correlations between the significantly high fatty acid metabolic genes in
186 UCDs and the significantly high *Burkholderia* transcript abundances, we conducted a Spearman

187 correlation analysis. All UCD normalized (by DESeq2) transcript counts that fell within the fatty
188 acid metabolism GO category by ErmineJ were used for this analysis. Also, UCD *Burkholderia*
189 normalized (by DESeq2) transcript abundance values from our previous research were obtained
190 (Rosales & Vega Thurber, 2015). A Spearman correlation analysis was then conducted with R
191 3.2.2 using function cor.test.

192

193 **Results:**

194 *Harbor seal brain transcriptome assembly*

195 In this study, we generated 11 harbor seal brain transcriptome libraries to distinguish
196 genes expressed in the brains of harbor seals during a PhV-1 infection and from an unknown
197 etiology. From here on, we will refer to PhV-1 infected samples as PhV-1 comparative or PhV-
198 1com as they were referred to as “comparative” in previous work (Rosales & Vega Thurber,
199 2015). From the 11 libraries, the Hi-Seq 2000 produced a total of 546,003,190 reads of 100bps in
200 length. Libraries ranged from 41,767,080 to 58,031,096 sequences, with means of 47,800,849
201 (SEM = 1,199,797) and 52,849,311 (SEM = 1,929,808) sequences, for UCD and PhV-1com
202 samples, respectively. The data showed no significant difference in the number of sequences
203 between PhV-1com and UCD datasets (Welch Two Sample t-test, $p = 0.07$).

204 To build the harbor seal transcriptome necessary for our downstream analyses, we used a
205 combination of a transcriptome guided approach with the *Leptonychotes weddellii* transcriptome
206 (NCBI accession: PRJNA232772) and *de novo* methods. The 11 libraries were aligned to the
207 *Leptonychotes weddellii* hypothetical transcriptome. A total of 163,769,951 sequences aligned
208 which equated to 27.43% of the total data. These sequences were then used for *de novo*
209 construction of the harbor seal transcriptome and is available on figshare

210 (<https://dx.doi.org/10.6084/m9.figshare.3581712.v1>). Next, each library was aligned to the
211 harbor seal *de novo* transcriptome with alignments ranging from 17.1% to 28.05% and there
212 were no significant differences between PhV-1com and UCD alignments (Welch Two Sample t-
213 test, p-value = 0.2825).

214 *UCD and PhV-1 infected seals show distinct gene expression profiles*

215 The transcriptome guided and *de novo* approach resulted in a harbor seal transcriptome of
216 32,856 transcripts. Next, the longest representative read was selected for each component and
217 29,512 transcripts remained with an average length of 269 bps. The maximum transcript length
218 was 54,385 bps, with a minimum length of 224 bps (Supplemental figure 1 A and B). Using
219 BLASTx, 25,840 (~87.5%) transcripts had significant similarity to proteins in the UniProt
220 database. A total of 1,962 differential expressed genes (DEG) were identified as measured by a
221 padj of ≤ 0.05 (data available on figshare <https://dx.doi.org/10.6084/m9.figshare.3767307.v1> and
222 <https://dx.doi.org/10.6084/m9.figshare.3766986.v1>). Datasets appear to have distinct gene
223 expression profiles, with UCD samples exhibiting tighter clustering than PhV-1com samples
224 (Fig. 1).

225 *Functional annotation of differently expressed genes distinguishes UCD from PhV-1 infected* 226 *harbor seals*

227 We explored enriched gene categories in the data by performing a gene ontology (GO)
228 analysis. In our pipeline, we identified 19,788 GO terms in the harbor seal transcriptome. After
229 filtering based on GO term p-values and multifunctionality (values generated by ErmineJ), 32
230 GO terms remained and from these terms the four most significantly enriched were: 1) “antigen
231 processing and presentation” (p-value = 1.00e-12), 2) “defense response to virus” (p-value =
232 1.00e-12), 3) “response to virus” (p-value = 1.00e-12), and 4) “innate immune response-

233 activating signal transduction” (p-value = 1.00e-05). After GO terms were semantically
234 summarized, the categories with the most significant GO terms were: 1) “antigen processing and
235 presentation” followed by 2) “response to amino acids”, 3) “DNA packaging”, and 4)
236 “mononuclear cell proliferation”. The least significantly enriched GO term categories were
237 “phagocytosis” and “fatty acid metabolism” (Fig. 2)

238 Genes that were significantly differentially expressed genes (DEGs; padj \leq 0.05) and
239 found in significantly GO enrichment analysis resulted in 112 significant genes that clustered
240 with their respective group (Fig. 3). The majority of transcripts (85.7%) in this analysis were up-
241 regulated in the PhV-1com samples. Transcripts that belonged to the fatty acid metabolism GO
242 category showed a higher gene expression in UCD samples (Fig. 3). Also, of particular interest,
243 GO categories for “defense response to virus” and “response to virus” were up-regulated in PhV-
244 1com and not UCD animals (Fig. 3). In addition, 3 out 4 PhV-1com samples showed gene
245 enrichment for bacterial infection, but a bacterial host response was not apparent in UCDs
246 (Supplemental Fig. 2).

247 *KEGG analysis reveals host responses to phocine herpesvirus-1 infection*

248 To further evaluate functional pathways found in UCD and PhV-1com disease states, we
249 annotated the translated harbor seal transcriptome with the KEGG Automatic Annotation Server
250 (KAAS). KAAS identified a total of 15,586 KOs from the whole transcriptome assembly and
251 from these we extracted the 1,464 DEGs. Using KegArray it was found that the five most
252 abundant KO pathways were for: Metabolic Pathways (107 members), PI3K-Akt Signaling
253 Pathway (43 members), Pathways in Cancer (39 members), Human T-Lymphotropic virus-1
254 Infection (36 members), and Herpes Simplex Infection (36 members). Given that PhV-1com
255 samples had previously been shown to have a herpesvirus infection (e.g., PhV-1), we focused on

256 the herpes simplex virus KEGG PATHWAY map and looked at genes up-regulated in PhV-
257 1com harbor seals. All 36 KO terms were up-regulated in PhV-1 infected samples and partially
258 mapped to the herpes simplex virus pathway (Fig. 4).

259 *Correlations of Burkholderia and UCD fatty acid genes*

260 We further evaluated transcripts assigned to fatty acid metabolism by GO enrichment
261 analysis. Transcripts that were significantly up-regulated in the fatty acid metabolism category
262 ($p_{adj} \leq 0.05$) in UCD animals were compared to KAAS annotation (Table 2). The transcript
263 annotations were similar using both the UniProt database and the KEGG GENES database
264 (Table 2). In addition, since UCD animals showed significant expression of fatty acids
265 metabolism and our earlier study showed significant levels of *Burkholderia* RNA we looked for
266 a correlation between these two factors (Rosales & Vega Thurber, 2015). A Spearman
267 correlation of the data yielded a significant correlation of fatty acid metabolism genes and
268 *Burkholderia* transcript abundance across the samples ($r_s = 0.809$ and a p -value = 0.004).

269

270 **Discussion:**

271 In marine mammals, transcriptomics has never been used to comprehend the cause of an
272 unknown disease and rarely has it been used to characterize the global gene expression of known
273 marine mammal stressors (Mancia et al., 2014; Niimi et al., 2014; Khudyakov et al., 2015a,b;
274 Fabrizio et al., 2016). Here, we used transcriptomics to compare gene expression patterns to
275 known and unknown disease states of stranded harbor seals. We infer the cause of a brain disease
276 in seven young harbor seals and characterize host pathways involved during a PhV-1 infection in
277 the brains of four young harbor seals.

278 *Gene expression of harbor seal brains with an unknown cause of death (UCD)*

279 As stated earlier, the initial hypothesis for the root cause of death of UCD harbor seals
280 was a viral infection. However, exposure to toxins, nutrient depletion, and hypoxia were also
281 candidates for the death of these animals. In our former work, we showed that a viral infection
282 was unlikely the cause of mortality in UCD harbor seals (Rosales & Vega Thurber, 2015). We
283 further confirmed this by demonstrating that GO categories for “defense response to virus” and
284 “response to virus” were not expressed in UCD animals (Fig. 3). At the same time, we validated
285 that UCD animals had a similar gene response at the time of death, thus supporting the notion
286 that these harbor seals died from the same disease (Fig. 1).

287 *UCD harbor seal gene response to bacteria*

288 In our previous work on this data set, we also found that there was a significant
289 abundance of *Burkholderia* transcripts in UCD animals and our new results indicate that these
290 same animals exhibit high fatty acid metabolic process gene expression (Fig. 3). In this study, we
291 found a significant correlation between *Burkholderia* and fatty acid genes. It is possible that fatty
292 acid metabolism is triggered by and/or provides an environment that promotes the growth of
293 *Burkholderia*, but substantial research needs to be conducted to confirm this correlation. To our
294 knowledge, there is no documentation of *Burkholderia* increasing due to high fatty acid
295 production, but there is evidence that *Burkholderia* can grow competitively in humans during
296 metabolically stressful situations (Schwab et al., 2014).

297 In addition, in this study, we found that significantly expressed DEGs for “response to
298 bacteria” were up-regulated in the majority of PhV-1 infected samples (3 out 4) and not
299 upregulated in UCDs. Since UCD samples had a significant abundance of *Burkholderia* it was
300 expected that UCD animals would have an upregulated gene expression to “response to bacteria”
301 (Supplemental figure 2). However, in our previous study we noted that the microbiome was

302 significantly less abundant in UCD animals when compared to PhV-1 infected animals. Thus it is
303 likely that the low abundance of bacteria in UCD animals compared to PhV-1 infected animals
304 drives this gene expression pattern.

305 *Fatty acid metabolism associated with harbor seals stranding*

306 To reiterate, in our GO summary analysis there was no indication of a viral infection, but
307 we did find “fatty acid metabolism” genes enriched in UCD animals. In fact, the DEGs analysis
308 demonstrated that this GO group was the most significantly up-regulated category in UCD
309 animals (Fig. 2 & 3). Using KEGG analysis, we further substantiated the involvement of these
310 genes in fatty acid metabolism (or related pathways involved in lipid and fat metabolism e.g.
311 steroid biosynthesis) (Table 2). Fatty acid metabolism genes are important for fundamental
312 cellular functions such as those involved in the formation of phospholipids and glycolipids, as
313 well as in the cell cycle energetics, like cell proliferation, differentiation, and energy storage.

314 In mammals, fatty acids are mostly acquired through dietary means except in the liver
315 and adipose tissue where fatty acid pathways are utilized (Kuhajda, 2000). In marine mammals,
316 esterified fatty-acids (NEFA) can be used as a proxy for nutritional health (Trites & Donnelly,
317 2003). For example, if gray seal pups fast for over a month they show elevated NEFAs and
318 reduced glucose (Rea et al., 1998). Although, given that UCD samples were in a rehabilitation
319 center and had normal weight measurements (Table 1), UCD harbor seals do not appear to have
320 died from starvation. However, high fatty acid gene activity, in regions other than the liver or
321 adipose tissues, can be symptomatic of metabolic diseases other than starvation. As an example,
322 cells with an up-regulation of fatty acid synthase (FAS) can be a sign of tumorigenesis (Kuhajda,
323 2000).

324 A possible mode of death for UCDs, is that these animal were unable to adequately take-
325 in nutrients since a lack of adequate dietary intake of some fatty acids can lead to an increase in
326 fatty acid metabolism in the brain (Innis, 2008). To illustrate, if an animal has an insufficient
327 intake of ω -3 fatty acids, then the brain increases in ω -6 fatty acid content. In a developing brain,
328 this increase in ω -6 fatty acids can lead to problems with neurogenesis, neurotransmitter
329 metabolism, and altered learning and visual function. Metabolic disorders are commonly
330 reported in cetaceans with hepatic lipidosis or fatty liver disease (Jaber et al., 2004).
331 Interestingly, UCD necropsies reported that four animals had hepatic lipidosis, which is a disease
332 attributed to toxins, starvation, or nutrient deprivation in weaning animals (Jaber et al., 2004).

333 Fatty acids, specifically, can be used to detect chemical or toxic stress in marine
334 organisms (Filimonova et al., 2016). Since, these animals did not appear to be starved, this
335 suggest that nutrient depletion or toxin exposure may have been involved in the die-off of UCDs
336 because (1) these were neonatal harbor seals, (2) the coincident description of the necropsy
337 reports, and (3) the fatty acid metabolic shifts in the brains of these animals. Other common
338 lesions found in UCDs were spleen hemosiderosis and spleen extramedullary hematopoiesis.
339 These syndromes have been associated with other metabolic diseases, but we are unsure if they
340 are directly related to high fatty acid gene expression in the brains of these neonatal seals.

341 As mentioned earlier, fatty-acid markers have been used to detect stress responses in
342 marine organisms (Trites & Donnelly, 2003; Filimonova et al., 2016). The transcripts detected in
343 this study have the potential to be used as biomarkers for stranded animals with an elusive
344 etiology or marine mammals that died from necrosis of the brain tissue. Gathering such
345 information may help in better understanding this mysterious disease and help properly diagnose
346 other animals.

347 *Gene expression of harbor seal brains infected with phocine herpesvirus-1*

348 The gene response of harbor seals infected with PhV-1 is mostly unknown, but our data
349 now illuminates some understanding of this interaction. The human Herpes simplex virus -1
350 (HSV-1) KEGG Pathway (Fig. 4) shows evidence that PhV-1 promotes some host gene
351 responses similar to other viruses in the subfamily *Alphaherpesvirinae*. Although, it appears that
352 there are still many pathways that differ between HSV-1 and PhV-1. However, it is likely that
353 PhV-1 host response may better parallel other viruses from its genus *Varicellovirus*, like bovine
354 herpesvirus -1 (BHV-1). In BhV-1, the host immune system has been shown to respond in three
355 stages: early cytokines, late cytokines, and cellular immunity or adaptive immunity (reviewed in
356 (Babiuk, van Drunen Littel-van den Hurk & Tikoo, 1996)). Although our data is non-temporal,
357 the summarized enriched GO analysis, illustrates evidence for aspects of each of these three
358 predefined temporal stages (Fig. 2). For example, “response to amino acid” (Fig. 2 pink blocks)
359 provides evidence of the cytokine immune responses found in stage 1. At the same time our
360 DEG analysis shows that Toll-like receptors (TLR) are significantly expressed in these animals
361 (p -value < 0.001); thus we speculate that TLR3 and TLR7 may be involved in the detection of
362 PhV-1 in harbor seal brain cells (Fig. 3). TLR7 is part of a TLR group that can detect viral
363 Pathogen- associated molecular patterns (PAMPs) within endosomes and lysosomes (Heil et al.,
364 2004) and TLR-3 is known to activate an antiviral state within an infected cell (Tabeta et al.,
365 2004). Thus we reason that TLR3 and TLR7 ultimately lead to the induction of a nonspecific
366 positive regulation inflammatory response seen in these animals (Fig. 2, pink blocks “positive
367 regulation of inflammatory response”).

368 Furthermore, cell chemotaxis, leukocyte chemotaxis, and interleukin-6 are also important
369 early cytokine stage responses found in our data (Fig. 2, pink blocks) (Babiuk, van Drunen

370 Littel-van den Hurk & Tikoo, 1996). Cell chemotaxis and leukocyte chemotaxis are needed for
371 recruitment of cells and could be responsible for attracting cells to the site of a PhV-1 infection,
372 while interleukin-6 promotes macrophage differentiation. Differentiated macrophages can then
373 secrete cytokines, like tumor necrosis factor (TNF) (Fig. 2. pink blocks). The early and late stage
374 cytokine activity is depicted in the summarized GO category “mononuclear cell proliferation”
375 (Fig. 2 yellow blocks). Once at the site of infection leukocytes are likely to proliferate, while late
376 stage cytokines can cause proliferation of mononuclear cells, such as T-cells, B cells, and
377 Natural Killer cells (NK cells).

378 The most pronounced category in this data is the last stage or cellular immunity (Fig. 2
379 orange blocks). Antigen processing and presentation is an important step in developing cellular
380 immunity, which occurs when an antigen, like PhV-1, is processed into proteolytic peptides and
381 loaded onto MHC class 1 or II molecules on a cell. We found that “antigen processing and
382 presentation” is a highly enriched GO category (p-value < 0.001) and that transcripts for MHC I
383 and II are highly expressed in PhV-1 infected samples (Fig. 3, $p_{adj} < 0.0001$), demonstrating that
384 the immune system of these young harbor seals was able to develop an adaptive immune
385 response to PhV-1. Furthermore, the “phagocytosis” category data, suggest that a cellular
386 mechanism to clear PhV-1 infected cells in harbor seals brains might be phagocytosis, as it was
387 an enriched GO category in these animals (Fig. 2, teal blocks). However, we cannot refute the
388 possibility that phagocytosis may be a route for viral entry into the cell. Recently in equine
389 herpes virus- (EHV-1), from the genus *Varicellovirus*, there was an indication of a phagocytic
390 mechanism for EHV-1 to enter some cells (Laval et al., 2016). Alternatively, or in conjunction,
391 PhV-1 may have appropriated the host exocytosis pathway to egress from the cell, as has been

392 noted in other alpha herpesviruses (Fig. 2, teal block “regulation of exocytosis”) (Hogue et al.,
393 2014).

394 *DNA packaging during a PhV-1 infection*

395 Another category enriched in our GO analysis was “DNA packaging” (Fig. 2, green
396 block), which occur when a chromatin structure is formed from histones to create nucleosomes
397 (Felsenfeld, 1978). Here, we predict that PhV-1 hijacked the host DNA packaging pathway.
398 Presently, there is controversy about the role of DNA packaging during herpesvirus infections.
399 Research shows that at least three different states of DNA packaging occurs during a herpesvirus
400 infection. Within the viral particle the double stranded genome is not packaged, but in the latent
401 state of the virus, it associates with cellular nucleosomes forming a cellular chromatin-like
402 structure (Lee, Raja & Knipe, 2016). The controversy arises from the lytic or replication cycle.
403 Studies show varying degrees of chromatin with herpesvirus DNA and these variations in
404 chromatin may be associated with viral transcription (Herrera & Triezenberg, 2004; Lacasse &
405 Schang, 2012; Lee, Raja & Knipe, 2016). We suspect that PhV-1 was either entering the latent
406 phase and/or that chromatin formation was occurring because of active viral transcription.

407 Of interest within the “DNA packaging” category the most significantly up-regulated
408 histone is H3.2 like protein (Fig. 3, p-value > 0.0001), a variant of histone H3. Some variants of
409 H3, like H3.3, have been shown to be important during herpesvirus transcription, the role of
410 H3.2 in herpesvirus is more elusive (Placek et al., 2009). Although, the role of H3.2 in the latent
411 phase cannot be disregarded since H3 has been associated with both the latent and lytic phases
412 (Kubat et al., 2004; Wang et al., 2005; Kutluay & Triezenberg, 2009). Our results suggest that
413 DNA packaging is important for PhV-1, but the exact role of DNA packaging in PhV-1 requires
414 further research.

415 *Caveats and considerations*

416 Marine mammal diseases can be difficult to diagnose given their protected status and the
417 challenge to gather conventional control samples for studies. For this research, we used two
418 disease states, one known and one unknown. Our study shows that this method can yield
419 valuable insight into host responses to infection, but we recognize the limitations to this
420 approach. For instance, we were limited to evaluating up-regulated genes and consequently, we
421 did not evaluate any down-regulated genes that may have been meaningful for understanding
422 these diseases.

423 In addition, it is probable that there are shared genes or pathways in both diseases, and
424 this commonality would not have been apparent in our DEG analysis between the two groups. As
425 an example, it is known that host fatty acids are also up-regulated during viral infections; thus
426 fatty acid DEGs in the UCD animals may actually have been even more numerous had we
427 compared UCD animals with a different group of animals that did not have a viral infection
428 (Jackel-Cram, Babiuk & Liu, 2007; Heaton et al., 2010; Spencer et al., 2011).

429 Finally, there is the potential that genes identified as up-regulated in one disease state are
430 actually a result of down-regulated genes in the other disease state. However, since we knew that
431 PhV-1 was an infectious agent in one cohort of animals and since we attained necropsy reports
432 with probable causes of UCD disease, we were able to confidently tease apart our results with
433 this information. Optimistically, with the increased use of HTS methods, we expect that more
434 transcriptome studies on marine mammals will become available and this may help diminish
435 these caveats.

436 In addition, it is apparent that the alignments rates in this study were low and this is likely
437 because alignments were conducted using the Weddell seal transcriptome and not the genome.

438 Using the Weddell seal genome, a greater portion of the data aligned (76.51%). These results are
439 in compliance with previous research where alignments to the transcriptome are lower than
440 aligning to the genome of an organism (Conesa et al., 2016). In spite of this, it is still a valid
441 approach to use the transcriptome with the caveat that novel genes are not likely to be identified
442 (Conesa et al., 2016). In this study, we did not use a genome-guided approach since this
443 method resulted in up to 1,323,851 transcripts, which is overly abundant. In addition, in the
444 genome-guided approach, only 4.9% of the data represented ORFs and the N50 score was 1,136.
445 However, the transcriptome guided method resulted in 32,856 transcripts with 65.9% of
446 transcripts accounting for ORFs and an N50 of 1,994 (S1 Table).

447 Moving forward, we like to acknowledge that we used a small sample size, especially for
448 PhV-1 infected animals (N=4). Including a larger sample size could elucidate other trends in
449 these diseases, such as the effects of gender (if any) or make correlations more apparent. In
450 addition, it is important to note that PhV-1 typically infects the adrenal glands of seals and the
451 infection does not always reach the brain (Gulland et al., 1997; Goldstein et al., 2005). Since
452 viruses infect organs differently, PhV-1 may not replicate in the same manner in the brain as it
453 does the adrenal glands. Future studies may focus on comparing transcriptomes from the brain,
454 adrenal glands, and other PhV-1 affected organs to determine any differences between the host
455 organs and virus interactions.

456 **Conclusion** 457

458 This is the first study to evaluate transcriptomes to better understand virus-host
459 interactions and brain tissue response to an unknown disease in marine mammals. In samples
460 with a PhV-1 brain infection, we identified pathways involved in innate and adaptive immunity,
461 as well as DNA packaging transcripts. We now have a better understanding of PhV-1 gene

462 expression in brain tissue of pinnipeds, which may lead to improved management and treatment
463 of PhV-1 infections. However, more work including time series data is needed to comprehend
464 the mechanism and progression of this disease. In addition, with this analysis, we were able to
465 further confirm our results, from our previous work, that UCD animals did not die from a viral
466 infection. Instead, we found that fatty acid metabolic genes were highly up-regulated in UCD
467 animals. It is unknown what may have caused a manifestation of fatty acid metabolism
468 dysregulation in the brains of these harbor seals, but it is probable that it may have been linked to
469 exposure to toxins or nutrient depletion.

470

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475

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- 661

Figure 1(on next page)

Batch effects on transcripts from the brain tissue of harbor seal samples.

Principal Coordinate Analysis (PCA) of all annotated transcripts in both PhV-1com and UCD harbor seals.

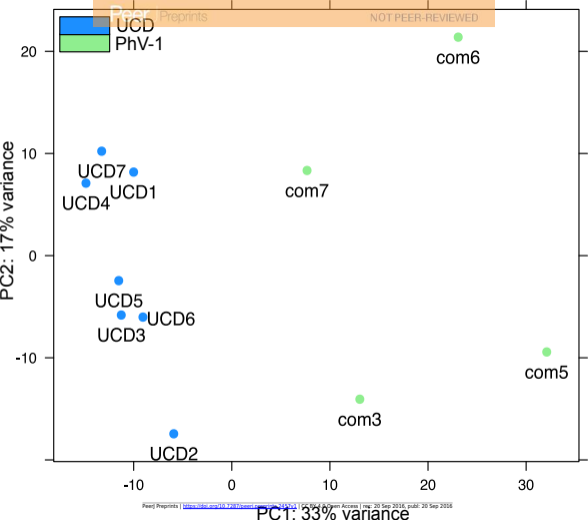


Figure 2 (on next page)

Semantically summarized GO terms.

Tree map summary of 32 significantly enriched GO terms (p -value ≤ 0.05 , and multifunctionality ≤ 0.85) of the whole transcriptome. The blocks are clustered by related terms and the size of the boxes are based on \log_{10} transformed p -values from GO enrichment analysis. Larger boxes represent more significant p -values.

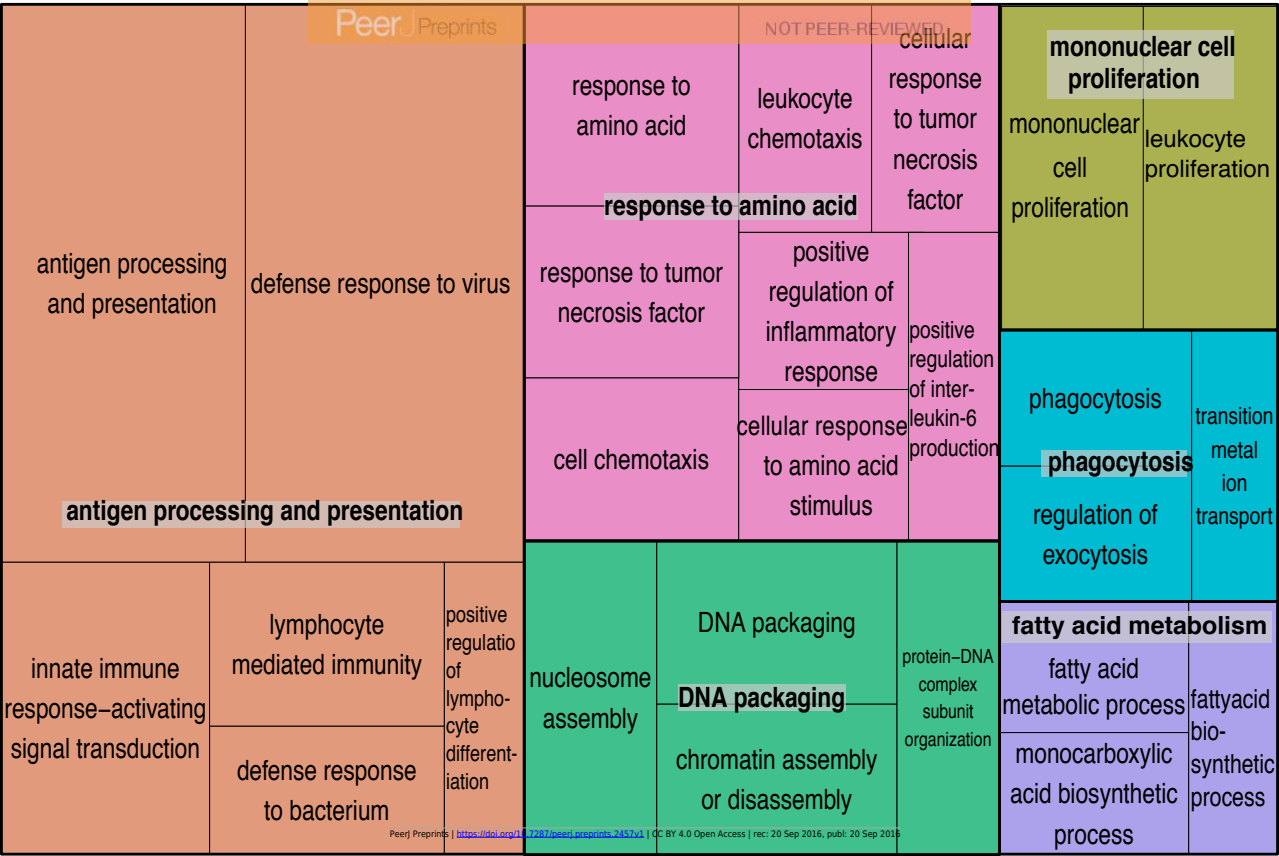


Figure 3(on next page)

Significant differentially expressed genes ($p_{adj} \leq 0.05$) within GO categories that were significantly enriched in the harbor seal transcriptome (GO p value ≤ 0.05 and multifunctionality of ≤ 0.85).

Heatmap of normalized gene counts expressed in rlog transformation (row z-score) from PhV1com and UCD harbor seals. Scatter plot of \log_2 fold change between PhV1 and UCD. The respective DEG p_{adj} values for each gene are represented by circles, with smaller circles denoting smaller p_{adj} values. Category: purple= GO term: fatty acid metabolic process, orange= GO terms: defense response to virus, and response to virus, grey= the other 29 GO terms that were significantly enriched in the harbor seal transcriptome.

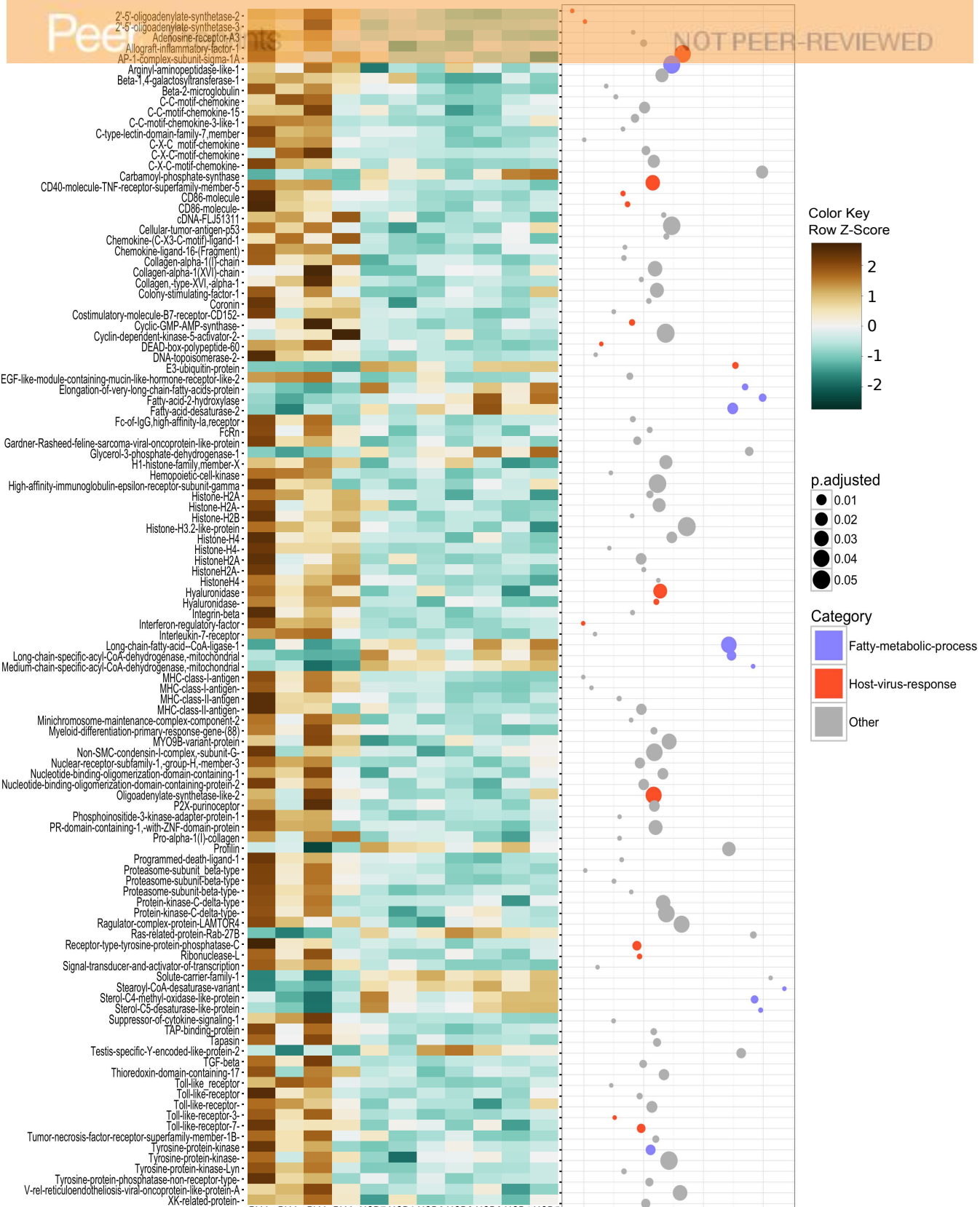


Figure 4(on next page)

KEGG pathway involved in human herpes-simplex-1 (HSV-1) showing similarities in host gene responses upon a PhV-1 infection.

Highlighted gray boxes represent terms that were significantly enriched DEGs in PhV-1 infected seals (DESeq2 $p_{adj} \leq 0.05$).

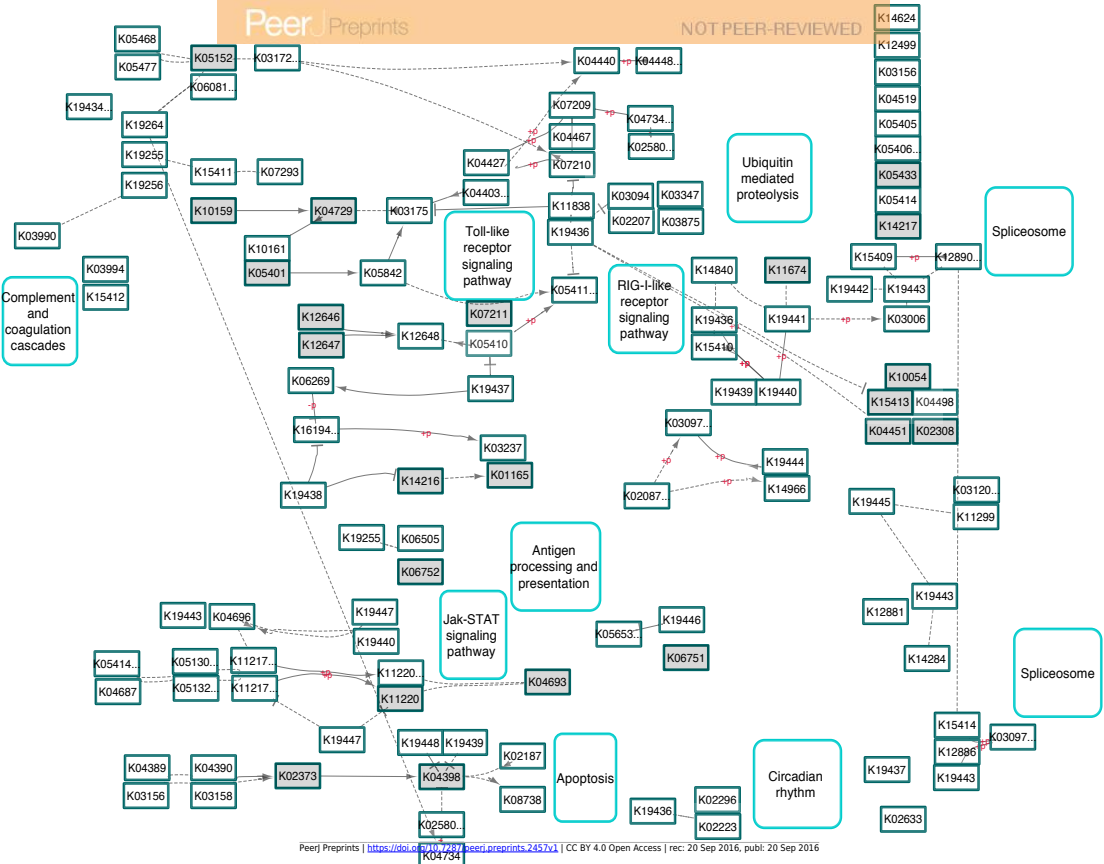


Table 1 (on next page)

Stranding information for harbor seal samples used in this study.

Sample ID	Date of stranding	Date of death	Date of necropsy	Common lesions	Age	Sex	Tissue	Weight in kilograms at necropsy
UCD1	4/8/09	7/1/09	7/2/09	Neuronal necrosis in the cortex and cerebellum, and hepatic lipidosis	Weaner	M	Cerebrum back	9.6
UCD2	4/9/09	7/26/09	7/29/09	Neuronal necrosis in the cortex and cerebellum, splenic hemosiderosis, spleen extramedullary hematopoiesis, and hepatic lipidosis	Weaner	F	Cerebellum front	11.0
UCD3	4/11/09	4/21/09	4/22/09	Neuronal necrosis in the cortex and cerebellum, splenic hemosiderosis, and spleen extramedullary hematopoiesis	Weaner	M	Cerebrum front	11.9
UCD4	4/17/09	7/6/09	7/6/09	Neuronal necrosis in the cortex and cerebellum, splenic hemosiderosis, spleen extramedullary hematopoiesis, and hepatic lipidosis	Weaner	F	Cerebrum front	13.0
UCD5	4/20/09	7/12/09	7/13/09	Neuronal necrosis in the cortex and cerebellum, and spleen extramedullary hematopoiesis	Weaner	F	Cerebrum front	10.8
UCD6	5/2/09	6/26/09	6/27/09	Neuronal necrosis in the cortex and cerebellum, splenic hemosiderosis, spleen extramedullary	Weaner	M	Cerebrum front	10.0

				hematopoiesis, and hepatic lipidosis				
UCD7	6/1/09	7/16/09	7/16/09	Neuronal necrosis in the cortex and cerebellum, splenic hemosiderosis, and spleen extramedullary hematopoiesis	Weaner	F	Cerebrum front	8.7
PhV-1com3	3/14/10	5/2/10	5/3/10	Necrosis in the liver, adrenal gland, and lymph tissue	Weaner	M	Cerebrum front	7.5
PhV-1com5	3/29/11	4/7/11	4/8/11	Hemorrhagic and congested lungs, mottled liver, congested meninges, intestinal necrosis, necrosis in the liver, and adrenal gland	Pup	M	Cerebrum front	7.5
PhV-1com6	4/16/11	4/24/11	4/25/11	Fat atrophy, omphalophebitis, enlarged mesenteric lymph nodes, thickened umbilicus, and necrosis in the liver and lung	Pup	M	Cerebrum front	11.0
PhV-1com7	5/25/12	5/25/12	5/26/12	Omphalophebitis, necrotizing splentitis, hepatitis, and adrenalitis	Pup	F	Cerebrum front/back	8.3

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Table 2 (on next page)

Transcripts in UCD samples involved in fatty acid metabolism.

Fatty acid metabolism transcripts that were significantly up-regulated (DEGs $p_{adj} \leq 0.05$) in UCD harbor seals and annotated using UniProt, GO terms, KEGG GENES and KO pathways.

PPAR = peroxisome proliferator-activated receptors, AMPK= adenosine monophosphate-activated protein kinase

Gene ID	UniProt annotation	GO category	KEGG annotation	KO pathway	Fold change	Padj
TR11985_c0	Elongation of very long chain fatty acids protein	Fatty acid metabolic process	Elongation of very long chain fatty acids protein 5	Fatty acid metabolism, biosynthesis of unsaturated fatty acids, and fatty acid elongation	0.697	0.001
TR13138_c0	Fatty acid 2-hydroxylase	Fatty acid metabolic process and fatty acid biosynthesis	4-hydroxysphinganine ceramide fatty acyl 2-hydroxylase	NA	0.99	0.003
TR5359_c0	Fatty acid desaturase 2	Fatty acid metabolic process and fatty acid biosynthesis	Fatty acid desaturase 2	PPAR signaling pathway, fatty acid metabolism, biosynthesis of unsaturated fatty acid, and alpha-Linolenic acid metabolism	0.49	0.011
TR15982_c0	Long-chain specific acyl-CoA dehydrogenase mitochondrial	Fatty acid metabolic process	Long-chain-acyl-CoA dehydrogenase	NA	0.469	0.007
TR7794_c0	Long-chain-fatty-acid--CoA ligase 1	Fatty acid metabolic process	long-chain acyl-CoA synthetase	Fatty acid biosynthesis, fatty acid degradation, fatty acid metabolism, PPAR signaling pathway, Peroxisome, and adipocytokine signaling pathway	0.424	0.036
TR9787_c0	Medium-chain specific acyl-CoA dehydrogenase mitochondrial	Fatty acid metabolic process	Acyl-CoA dehydrogenase	Fatty acid metabolism, PPAR signaling pathway, Carbon metabolism, beta-Alanine metabolism, valine, leucine isoleucine degradation, Fatty acid	0.831	2.37E-06

				degradation, and propanoate metabolism		
TR283_c0	Stearoyl-CoA desaturase variant (Fragment)	Fatty acid metabolic process and fatty acid biosynthesis	Stearoyl-CoA desaturase	AMPK signaling pathway, fatty acid metabolism, PPAR signaling pathway, biosynthesis of unsaturated fatty acids, and longevity regulating pathway - worm	1.354	1.28E-13
TR1355_c0	Sterol-C4-methyl oxidase-like protein (Fragment)	Fatty acid metabolic process	Methylsterol monooxygenase	Steroid biosynthesis	0.856	0.003
TR10658_c1	Sterol-C5-desaturase-like protein (Fragment)	Fatty acid metabolic process and fatty acid biosynthesis	Delta7-sterol 5-desaturase	Steroid biosynthesis	0.957	9.69E-05

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