

# 1 **Host age shapes virome abundance and diversity in birds**

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3 **Running Title:** Host age impacts avian viromes

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20 **Abstract**

21 Host age influences the ecology of many microorganisms. This is evident in one-host – one virus  
22 systems, such as influenza A virus in Mallards, but also in community studies of parasites and  
23 microbiomes. We used a meta-transcriptomic approach to assess whether host age is associated with  
24 differences in the abundance and diversity of avian viromes. We used samples from cohabiting  
25 Ruddy Turnstones (*Arenaria interpres*) across three age groups, collected at two contrasting points  
26 in their annual migratory cycle. Overall, we identified 14 viruses that likely infect birds, 11 of  
27 which were novel, including members of the *Reoviridae*, *Astroviridae*, *Picornaviridae*, and  
28 *Phenuiviridae*. Strikingly, 12 of the viruses identified were from juvenile birds sampled in the first  
29 year of their life, compared to only two viruses in adult birds. Similarly, both viral abundance and  
30 alpha diversity were higher in juvenile than adult birds. Notably, time of sampling had no  
31 association with virome structure such that the migratory period may not play a major role in  
32 structuring avian viromes. As well as informing studies of virus ecology, that host age impacts viral  
33 assemblages is a critical consideration for the future surveillance of novel and emerging viruses.

## 34 **Introduction**

35 Vertebrate animals of different ages also differ in their exposure and susceptibility to  
36 microorganisms [1]. The importance of host age for the epidemiology and ecology of  
37 microorganisms has been demonstrated by differences in the diversity and prevalence of parasites  
38 [2], bacteria [3, 4], and viruses [5-8] among hosts of different ages. In the case of wild birds, host  
39 age is associated with a higher prevalence of viruses in juvenile compared to adult birds. These  
40 include beak-and-feather disease virus in parrots [9], avian pox virus in albatross and tits [10, 11],  
41 avian avulavirus-1 in cormorants and waterfowl [12, 13], infectious bronchitis virus in poultry [14]  
42 and influenza A virus in wild waterfowl [8].

43 Despite a similar potential for exposure, viral susceptibility varies among adult and juvenile birds.  
44 This is believed to be due to an increase in the immune repertoire with age, with the age-related  
45 accumulation of immunity to influenza A virus in Mute swans (*Cygnus olor*) an informative case in  
46 point [15]. This increase of immunity in adults, coupled with juvenile immunologically naïve birds  
47 entering populations, may explain the significant increase in the prevalence of influenza A virus in  
48 juveniles compared to adults [7, 8], regardless of the time point of sampling [16-21]. This is  
49 evident when repeatedly sampling sentinel ducks, in which individuals have multiple influenza A  
50 virus infections in the first 6 months of age, compared to no or only short infections in the  
51 subsequent year of life, despite similar viral exposure [22]. In addition to varying susceptibility,  
52 exposure to viruses may similarly vary in wild birds. For example, in migratory birds such as  
53 shorebirds, adults and juveniles undertake southward migrations at different times, and in some  
54 species use markedly different routes, resulting in different virus exposure potential [23, 24].

55 As host age impacts the presence and prevalence of a range of specific viruses, we ask here whether  
56 host age is also associated with modulating communities of viruses carried by birds? To this end,  
57 we used the virome-scale data obtained from metagenomic next-generation sequencing as means to  
58 go beyond the “one-host, one virus” model and provide key data on the abundance and diversity of  
59 *all* the viruses present in a particular species. We used Ruddy Turnstones (*Arenaria interpres*) as a  
60 model species as they are known to be important reservoir hosts for viruses such as influenza A  
61 virus as well as for an abundance of other viruses [25, 26]. In the East Asian-Australasian Flyway,  
62 this species breeds in Arctic Siberia and following migration, spends its non-breeding period along  
63 the shorelines of Australia. Accordingly, we sampled individuals across three age groups at two  
64 different points in their annual cycle, just after migration, upon arrival in Australia and again shortly  
65 before departure to their Arctic breeding grounds. From these data we demonstrate that juvenile  
66 birds have significantly higher viral abundance and diversity compared to adult birds and that  
67 different time points in their migratory cycle had no apparent effect on virome structure.

## 68 **Methods**

### 69 *Ethics statement*

70 This research was conducted under approval of the Deakin University Animal Ethics Committee  
71 (permit numbers A113-2010 and B37-2013). Banding was performed under Australian Bird  
72 Banding Scheme permit (banding authority number 8001). Research permits were approved by the  
73 Department of Primary Industries, Parks, Water and Environment, Tasmania (permit number FA  
74 13032).

### 75 *Sample collection*

76 All birds were captured and sampled as part of a long-term study program on King Island,  
77 Tasmania, Australia (39°55'52"S, 143°51'02"E). Birds were captured by cannon netting at two time  
78 points: November 2014 following arrival from migration, and March 2015 prior to departure for  
79 migration to Siberia. Birds were aged using plumage characteristics and divided into three age  
80 categories: (i) 1o - juvenile birds hatched the previous breeding season, (ii) 2o – adolescent birds in  
81 their second year of life that overwintered on King Island (these birds could only be identified in  
82 November) and (iii) 3+ - adult birds, classified as those that are 3 years and older (Fig 1). A  
83 combination of oropharyngeal and cloacal samples were collected using a sterile tipped applicator  
84 and placed in virus transport media (VTM; Brain-heart infusion broth containing 2 × 10<sup>6</sup> U/L  
85 penicillin, 0.2 mg/ml streptomycin, 0.5 mg/ml gentamicin, 500 U/ml amphotericin B, Sigma).

### 86 *RNA library construction and sequencing*

87 RNA library construction, sequencing and RNA virus discovery was carried out as described  
88 previously [25, 27]. Briefly, RNA was extracted from swabs using the MagMax *mirVana*<sup>TM</sup> Total  
89 RNA isolation Kit (ThermoFisher Scientific) on the KingFisher<sup>TM</sup> Flex Purification System  
90 (ThermoFisher Scientific). Extracted samples were assessed for RNA quality using the TapeStation  
91 2200 and High Sensitivity RNA reagents (Aligent Genomics, Integrated Sciences). The 10 samples  
92 with the highest concentration were pooled using equal concentrations and subsequently  
93 concentrated using the RNeasy MinElute Cleanup Kit (Qiagen) (Table S1).

94 Libraries were constructed using the TruSeq total RNA library preparation protocol (Illumina) and  
95 rRNA was removed using the Ribo-Zero-Gold kit (Illumina). Paired-end sequencing (100bp) of the  
96 RNA library was performed on the HiSeq2500 platform. All library preparation and sequencing was  
97 carried out at the Australian Genome Research Facility (AGRF, Melbourne). All reads have been  
98 deposited in the Sequence Read Archive (SRA; BioProject XXX).

### 99 *RNA virus discovery*

100 Sequence reads were demultiplexed and trimmed with Trimmomatic followed by *de novo* assembly  
101 using Trinity [28]. No filtering of host/bacterial reads was performed before assembly. All  
102 assembled contigs were compared to the entire non-redundant nucleotide (nt) and protein (nr)  
103 database using blastn and diamond blast [29], respectively, setting an e-value threshold of  $1 \times 10^{-10}$  to  
104 remove potential false-positives.

105 Abundance estimates for all contigs were determined using the RSEM algorithm implemented in  
106 Trinity. All viral contigs that returned blast hits with paired abundance estimates were filtered to  
107 differentiate those with invertebrate [30], lower vertebrate [31], plant or bacterial host associations  
108 using the Virus-Host database (<http://www.genome.jp/virushostdb/>). The list was further cross-  
109 referenced against a known list of viral contaminants [32].

#### 110 *Virus genome annotation and phylogenetic analysis*

111 Contigs greater than 1000bp in length were inspected using Geneious R10 (Biomatters, New  
112 Zealand), and open reading frames (ORF) corresponding to predicted genome architectures based  
113 on the closest reference genomes were interrogated using the conserved domain database (CDD,  
114 <https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) with an expected e-value threshold of  $1 \times 10^{-5}$ .  
115 Reads were subsequently mapped back to viral contigs using bowtie2 [33]. Viruses with full-  
116 length genomes, or incomplete genomes possessing the full-length RNA-dependant RNA  
117 polymerase (RdRp) gene, were used for phylogenetic analysis. Briefly, amino acid sequences of the  
118 polyprotein or gene encoding for the RdRp were aligned using MAFFT [34], and gaps and  
119 ambiguously aligned regions were stripped using trimAL [35]. The best-fit model of amino acid  
120 substitution was then determined for each data set, and maximum likelihood trees were estimated  
121 using IQ-TREE [36]. In the case of coronaviruses, avastroviruses, and avian avulavirus,  
122 phylogenies were also estimated using the nucleotide sequences of complete or partial reference  
123 genome sequences to better place viruses in context of currently described avian viral diversity.  
124 Novel viral species were identified as those that had <90% RdRp protein identity, or <80% genome  
125 identity to previously described viruses. Newly identified viruses were named after shipwrecks  
126 surrounding King Island, Tasmania. Genome sequences have been deposited in NCBI GenBank  
127 (accession numbers XX-XX).

#### 128 *Viral diversity and abundance across libraries*

129 Relative virus abundance was estimated as the proportion of the total viral reads in each library  
130 (excluding rRNA). All ecological measures were estimated using the data set comprising viruses  
131 associated with birds and mammals, albeit with all retroviruses and retrovirus-like elements

132 removed (hereafter, “avian virus data set). Analyses were performed using R v 3.4.3 integrated into  
133 RStudio v 1.0.143, and plotted using *ggplot2*.

134 Both the observed virome richness and Shannon effective (alpha diversity) were calculated for each  
135 library at the virus family and genus levels using modified Rhea script sets [37], and compared  
136 between avian orders using t-tests. Beta diversity was calculated using the Bray Curtis dissimilarity  
137 matrix and virome structure was plotted as a function of nonmetric multidimensional scaling  
138 (NMDS) ordination and tested using Adonis tests using the *vegan* [38] and *phyloseq* packages [39].

139 To mitigate the reporting of false-positives due to metagenomic index-hopping, contigs were  
140 assumed to be the result of contamination from another library if the read count representing the  
141 abundance was less than 0.1% of that representing the highest count for that virus among the other  
142 libraries. Short, low abundance contigs from viral genera not detected in any other library  
143 sequenced on the same lane were retained.

144

## 145 **Results**

### 146 *Overall virus abundance and diversity*

147 We characterized the total transcriptomes of Ruddy Turnstones from different age categories upon  
148 arrival and prior to departure of migration (Fig 1, Table S1). All individuals were captured in  
149 March/November, regardless of age category, and on the same beaches on King Island, Tasmania.

150 There was a large range in total viral abundance (0.16– 9.91% viral reads) and putative avian viral  
151 abundance (0.0071– 1.13% viral reads) in each library (Table S1, Fig. S1). In addition to viral reads  
152 that were likely associated with *bona fide* avian viruses, libraries had numerous reads matching  
153 invertebrate, plant and bacterial viruses as well as retroviruses (Fig. S1). Although these  
154 retroviruses are likely associated with birds, the challenge of differentiating between endogenous  
155 and exogenous retroviruses meant that they were excluded from the analysis, as were those viruses  
156 most likely associated with arthropods, plants, and bacteria. In total, 11 of the 14 viruses identified  
157 in this study likely represent novel avian viral species (Table S2, Fig. 2). Novel species were  
158 identified in the double-stranded RNA viruses (*Reoviridae*, genus *Rotavirus* and genus  
159 *Orthoreovirus*), positive-sense single-stranded RNA viruses (*Astroviridae*, *Picornaviridae* genera  
160 *Hepatovirus*, *Gallivirus*, and unassigned genera), and negative-sense single-stranded RNA virus  
161 which is potentially an arbovirus (*Bunyavirales*, *Phenuiviridae*).

### 162 *Novel double-stranded RNA viruses*

163 We identified two novel viruses in two genera of the family *Reoviridae* - *Rotavirus* and  
164 *Orthoreovirus*. Maypo virus, a *Rotavirus*, was at very low abundance (0.01% reads) and no full  
165 segments were identified with the exception of VP7 that was used for classification within rotavirus  
166 species. Maypo virus was separated by a long branch from a clade containing rotavirus G, B, I and  
167 viruses identified previously in metagenomic studies (Fig S2). Cataraqvi virus, an *Orthoreovirus*,  
168 fell as a sister lineage to a recently described avian orthoreovirus - Tvarminne virus (Fig 3A).  
169 Cataraqvi virus was detected in two libraries - juvenile (1o) and adolescent (2o) birds sampled  
170 concurrently - with similar abundance levels (0.5% and 0.25% of reads in each library, respectively)  
171 showing viral sharing across these age groups (Fig 3A, Fig 6). The two genomes recovered were  
172 highly similar, with only ~1 nucleotide difference in each of the lambda segments. While we could  
173 not assemble this genome from adult 3+ birds, contigs of Cataraqvi virus were also found,  
174 comprising 0.0023% of the library. As this represents >0.1% of the read abundance of the library in  
175 which this virus is at its highest abundance we tentatively exclude index-hopping.

#### 176 *Novel single-stranded RNA viruses*

177 Three novel avastroviruses were identified in this analysis, two in the library of 1o birds sampled in  
178 March and one in the 2o birds sampled in November. Specifically, although Neva virus and  
179 Carnarvon virus occupy different phylogenetic positions, both are related to viruses previously  
180 classified as avastrovirus 2 (which includes avian nephritis virus), while Blencathra virus is a sister  
181 lineage to an astrovirus recently described in the pink-eared duck [40] (Fig 3B, Fig S3).

182 We also identified five picornaviruses (family *Picornaviridae*). Three of these - Waterwitch virus,  
183 Whister virus, and Loch Leven virus – fall as sister lineages to members of the genus *Hepatovirus*  
184 and *Tremovirus* and were found exclusively in 1o birds, with Waterwitch virus and Whister virus  
185 forming a distinct clade (Fig S4). In contrast, British Admiral virus fell as a divergent sister-group  
186 to the *Gallivirus* genera, while Netherby virus, the only picornavirus found in a 3+ bird, fell into a  
187 currently unassigned clade comprising a chicken and a quail phacovirus (Fig S4).

188 We also identified a novel virus in the family *Phenuiviridae*, Bruthen virus. This virus fell in a  
189 group of unclassified viruses that are the sister-group to members of the genus *Phlebovirus* that  
190 contains numerous tick-borne viruses (Fig 4). In the phylogeny Bruthen virus is most closely related  
191 to Laurel Lake virus sampled from *Ixodes scapularis* ticks in the USA [41]. However, because of  
192 the long branch lengths across the tree as a whole, including that leading to Bruthen virus, it is  
193 currently unclear whether this virus is a strictly arthropod virus or an arthropod-borne virus that  
194 may infect birds (Fig 4).

195



196 *Identification of previously described avian viruses*

197 We identified two members of the *Coronaviridae* – Duck coronavirus 2714 (genus  
198 *Gammacoronavirus*) and a virus here referred to as Shorebird deltacoronavirus (genus  
199 *Deltacoronavirus*) (Fig 5, Fig S5-S6). These viruses are common in wild waterbirds, although most  
200 sequence data is limited to short fragment of the RdRp (Fig S8, S9) [42]. In the ORF1ab phylogeny,  
201 the Duck coronavirus 2714 sequence (subgenus *Igacovirus*) was most closely related to a sequence  
202 identified in gulls in Canada [43], however based on a phylogeny comprising 400bp of the RdRp,  
203 the sequence revealed here fell in a clade dominated by viruses previously identified in Australia,  
204 and most closely related to viruses identified in Ruddy Turnstones from King Island, sampled in  
205 2016 (Fig 5, Fig S5) [44]. In the ORF1ab phylogeny the Shorebird deltacoronavirus fell as a sister  
206 lineage to the subgenus *Buldacovirus*. Based on a phylogeny comprising 400bp of the RdRp, for  
207 which more sequences are available, this virus was most closely related to viruses previously  
208 identified in Australia, including shorebirds from Victoria and Ruddy Turnstones from King Island  
209 in 2016 (Fig S6) [44]. The genome of Shorebird deltacoronavirus revealed here comprises the first  
210 full genome of this putative viral species, and the detection of two different coronaviruses from  
211 these birds suggests Ruddy Turnstones may be important reservoirs for both gamma and  
212 deltacoronaviruses.

213 The variant of avian avulavirus 2 identified here (AAvV-2/Ruddy turnstone/Tasmania/2015) was a  
214 (weakly supported) sister-group to a clade of viruses described in wild birds in China (Fig S7), and  
215 we speculate that this likely represents a lineage that is more common in wild birds than poultry.  
216 Unlike the case of avian avulavirus 1 (Newcastle Disease), the polybasic cleavage site within the F  
217 gene is not a determinant of the pathogenicity of avian avulavirus-2 in chickens [45, 46]: as such, we  
218 have not performed cleavage site annotation. This virus had a very high abundance in the library  
219 comprising adult (3+) birds sampled in March (comprising 99% of all avian viral reads and 0.2% of  
220 all reads). Reads for this virus were also identified in the juvenile (1o) birds simultaneously  
221 sampled, and adolescent (2o) birds sampled in November, although with very low abundance  
222 (0.00079% reads and 0.00014% reads, respectively, but above the assumed index-hopping threshold  
223 of 0.1% of the most abundant virus).

224 *Age affects both virome diversity and abundance*

225 Regardless of sampling period, juvenile (1o) birds had higher viral abundance and diversity than  
226 adult (3+) birds at the viral family, genus and species levels. First, viral abundance was higher in  
227 juvenile than adult birds, with a 10-fold difference in November (1.12% compared to 0.007% avian  
228 viral reads) and March (0.2% compared to 0.02% avian viral reads). Adolescent (2o) birds, which  
229 overwintered on the island rather than making a return trip to the Arctic breeding grounds like the



230 3+ birds did, also had very high viral abundance in November (0.7% avian viral reads) (Fig 6A).  
231 Second, this trend remained when assessing diversity within each sample (i.e. alpha diversity).  
232 Observed richness and Shannon diversity was higher in juvenile than adult birds, in which observed  
233 richness was significantly higher in 10 birds at the viral genus level (Fig S8) ( $t = 5.8138$ ,  $df =$   
234  $1.4706$ ,  $p = 0.049$ ). At the viral species level, there was a significant difference in the number of  
235 viral species in juvenile compared to adult birds ( $t = 9$ ,  $df = 1$ ,  $p = 0.05$ ): in November and March we  
236 identified five and six viruses in juvenile birds, respectively, compared to one virus from each of the  
237 libraries comprising adult birds, with adolescent birds exhibiting intermediate values (two virus  
238 species described) (Fig 2, Fig 6). Although this study is clearly limited in sample size, the overall  
239 trend remains across our biological replications (here different time periods). Sampling period was  
240 not a significant predictor of either observed richness or Shannon diversity at the virus family,  
241 genus and species levels. For example, when assessing the number of viral species, we observed the  
242 same number of viruses revealed (excluding the adolescent 20 birds as they were only sampled in  
243 November) in both November ( $n=6$ ) and March ( $n=7$ ) (Fig 2).

244 To ensure this trend was not due to a sequencing artefact we tested for differences in viral  
245 abundance and alpha diversity in those viruses sampled from birds but that most likely infected  
246 invertebrate hosts. These invertebrate viruses effectively comprise a null model: because they do  
247 not actively replicate in birds we would expect to see similar abundance levels in both the juvenile  
248 and adult birds. As expected, there was no statistical difference between juvenile and adult birds ( $t$   
249  $= 1.0022$ ,  $p = 0.5$ ). Furthermore, juvenile and adult birds exhibited similar levels of both alpha  
250 diversity measures for invertebrate viruses (observed richness or Shannon diversity), regardless of  
251 whether the measure was performed at the virus “cluster” level [30] (Observed Richness  $t = -$   
252  $1.3416$ ,  $p = 0.3499$ ; Shannon Diversity  $t = -0.6925$ ,  $p = 0.5606$ ) or at the virus family level (and  
253 family-like when no family classification was available) (Observed Richness  $t = 0.56569$ ,  $p =$   
254  $0.6693$ ; Shannon Diversity  $t = -0.54246$ ,  $p = 0.6425$ ) (Fig S9).

255 No obvious trends were observed with respect to beta diversity (Fig S10). Specifically, neither age  
256 ( $R^2=0.49$ ,  $p=0.33$ ) nor sampling time point ( $R^2=0.2$ ,  $p=1$ ) was associated with avian virome  
257 structure at the viral genus or family levels. There was very limited viral sharing (i.e. connectivity)  
258 among birds of different age categories even though they were sampled on the same beaches at the  
259 same time. The only viral species that we were able to annotate from more than one library was  
260 Cataraqui virus, identified in juvenile and adolescent birds at the November time point: however,  
261 reads for this virus were also present in the adult birds in November (Fig 2, Fig 3A). While the full  
262 genome of avian avulavirus-2 was only identified in the adult birds in March, reads were present in  
263 juvenile birds from March. Despite this, due to cohabitation, virus connectivity was far lower than

264 expected. We suggest that adult birds may already have antibodies against many of these viruses,  
265 resulting in no detectable infections in this age category.

266

## 267 **Discussion**

268 We used a meta-transcriptomic approach to help reveal the role of host age in shaping the avian  
269 virome. Through this, we identified 14 viruses, of which 11 are novel, in apparently healthy wild  
270 migratory birds from a single sampling location in Australia. The largest diversity of viruses were  
271 from the families *Astroviridae*, *Coronaviridae*, *Picornaviridae* and *Reoviridae*, from which more  
272 than one putative virus species was identified in each case.

273 Based on studies of the ecology of both avian influenza A virus [1, 7, 8], and more recently, of virus  
274 communities in bats [47], we predicted that juvenile birds would have high viral abundance and  
275 diversity compared to adult birds. During the non-breeding period, Ruddy Turnstones on King  
276 Island, Tasmania spend time in small (~50 birds) groups comprising both adult and juvenile birds,  
277 and are often restricted to small stretches of coastline (~1 km). Importantly, our sampling collection  
278 and selection strategy means that within each sampling period (November, and March) we compare  
279 birds of different age groups that are cohabiting, enabling us to identify any viruses that are shared  
280 between age groups within a given sampling period. Indeed, we observed high viral abundance and  
281 diversity in juvenile birds compared to very low abundance and diversity in adult birds. Adolescent  
282 birds shared a viral species, Catarauqui Virus, with co-sampled juvenile birds, demonstrating a  
283 degree of viral sharing among these two age categories. The high viral diversity and abundance in  
284 juvenile birds, but low levels in conspecific adult birds, strongly suggests that adult birds have  
285 immunity against these viruses. As a consequence, our results raise important questions about the  
286 robustness and longevity of antibodies against viruses in wild birds, such as the Ruddy Turnstone.  
287 Work on avian influenza A viruses suggests that short-lived species, such as dabbling ducks, may  
288 have short lived antibodies [48] and may therefore be reinfected many times [49], in comparison to  
289 long lived species such as swans [15] and shearwaters [50] that had detectable, neutralizing  
290 antibodies for years. Our data suggest that Ruddy Turnstones may fit into the latter category,  
291 although this needs to be clarified by antibodies studies.

292 Due to limited sample size, we utilized a biological replicate to test the impact of age. Specifically,  
293 we sampled birds following their arrival on King Island and prior to their departure following their  
294 non-breeding staging period. Despite only being four months apart, we found no evidence of viral  
295 connectivity between these two time points. This clearly demonstrates the transient nature of the  
296 avian virome, in which there was no consistent detection of viral species across sampling points.

297 Further, we found no difference in viral abundance or diversity of adult or juvenile birds when  
298 comparing those captured in November (recently arrived) or the subsequent March (imminently  
299 prior to departure of the 3+ adult birds). This not only shows the lack of a migratory cycle effect,  
300 but also that the impact of age is strong, despite potentially large differences in physiology of  
301 Ruddy Turnstones at these sampling events. Prior to migration, birds go through a period of  
302 impressive mass increases; some species may more than double their body mass, with a 1-3%  
303 increase per day [51, 52]. Indeed, birds captured in March had an additional 50% in mass compared  
304 to November. Studies in the microbiome of migratory birds found that the majority of bacterial taxa  
305 are not affected by migration when comparing resident birds and those under active migration  
306 immediately upon arrival [53]. Other changes during active migration may be related to immune  
307 responses. It has been suggested that migrating animals should boost their immune function during  
308 migration [54, 55]. A wind tunnel experiment found no difference in immune function between  
309 “migrating” Red Knots (*Calidris canutus*) and control birds [56]. In contrast, migratory Common  
310 Blackbird *Turdus merula* individuals had a lower innate immune function during migration  
311 compared with resident individuals [57]. Despite the different migration dispositions between the  
312 November and February time points, we found a consistent age effect and no difference in  
313 abundance, alpha diversity or beta diversity across time points. We also confirmed that this age  
314 effect was not an artefact by using invertebrate-associated viruses, that do not replicate in birds, as  
315 an intrinsic biological control. Importantly, we found that invertebrate associated viruses  
316 demonstrated no differences in viral abundance or alpha diversity in juvenile compared to adult  
317 birds, in marked contrast to the pattern in ‘true’ avian viruses. This observation is consistent with  
318 previous studies of virus ecology, in which influenza A viruses exhibit higher prevalence in  
319 juveniles compared to adults [7, 8], and juveniles have a greater number of infections and diversity  
320 of subtypes over an autumn season compared to adult birds [22]. Similarly, caves hosting a larger  
321 proportion of juvenile bats had a larger viral diversity as compared to those hosting a low  
322 proportion of juvenile bats [47].

323 Our results allow us to reflect on the potential barriers to migration, specifically why some  
324 individuals remain on non-breeding areas and do not migrate. Annual migrations are undertaken by  
325 adult shorebirds, that also have with lower viral abundance and diversity. We can therefore  
326 hypothesize that one of the reasons adolescent birds do not undertake a northward migration in their  
327 second year may be increased virus susceptibility, itself due to lower age-dependant immunity.  
328 Here, we show high viral abundance in both juvenile and adolescent birds. Even without obvious  
329 disease, the infection status and intensity of some viral infections may have negative effects on  
330 body stores, refuelling capacity, movement, phenology and survival [58]. These effects may result

331 in “migratory culling” - the combined physiological effects of migration and infection mitigation  
332 that may remove individuals from the population [59]. This hypothesis may be central to  
333 understanding an important barrier to migration of juvenile and adolescent birds, given  
334 demonstratively high viral abundance and diversity compared to adult conspecifics, and clearly  
335 merits further study.

336 Overall, we demonstrate a large viral diversity in Ruddy Turnstones and markedly different viromes  
337 that were detected in birds across age groups and between sampling periods. This, in turn,  
338 highlights the transient nature of the avian RNA virome and the snap-shot nature of virome studies  
339 to date. Both these characteristics have recently been observed in studies of bat viromes [47],  
340 strongly suggesting this is an important consideration in all ecological studies that utilise virome  
341 data. Beyond ecological studies, this is a critical consideration for future surveillance efforts for  
342 novel and emerging viruses. Finally, we further demonstrate that apparently healthy wild birds are  
343 able to sustain high viral loads and diversity without obvious signs of disease. This, combined with  
344 the continued detection of viral families previously associated with diseases such as gastroenteritis  
345 or hepatitis [60, 61], suggests that many of the viruses may not be pathogens.

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354

## 355 **Competing interests**

356 The authors declare that they have no conflict of interest.

357

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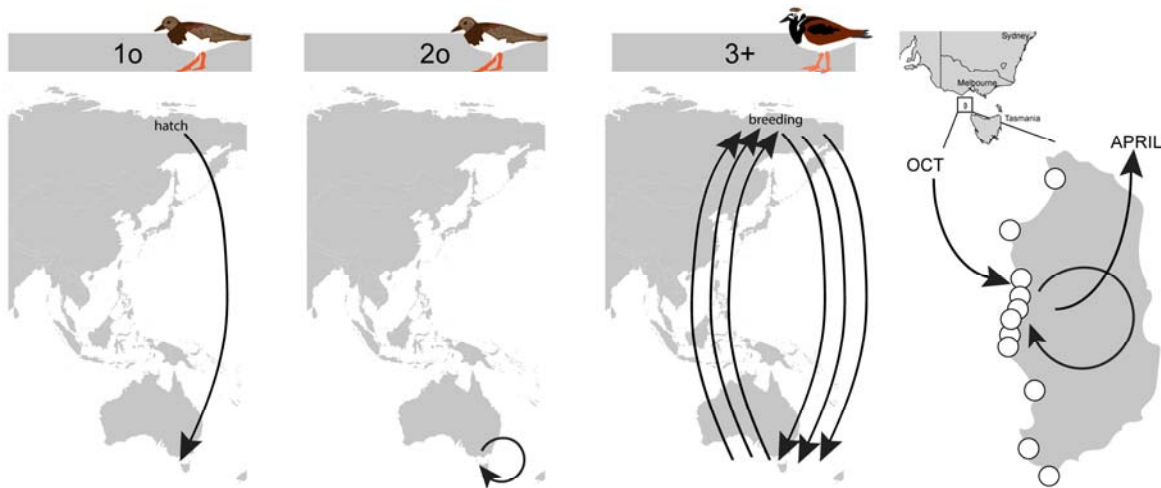


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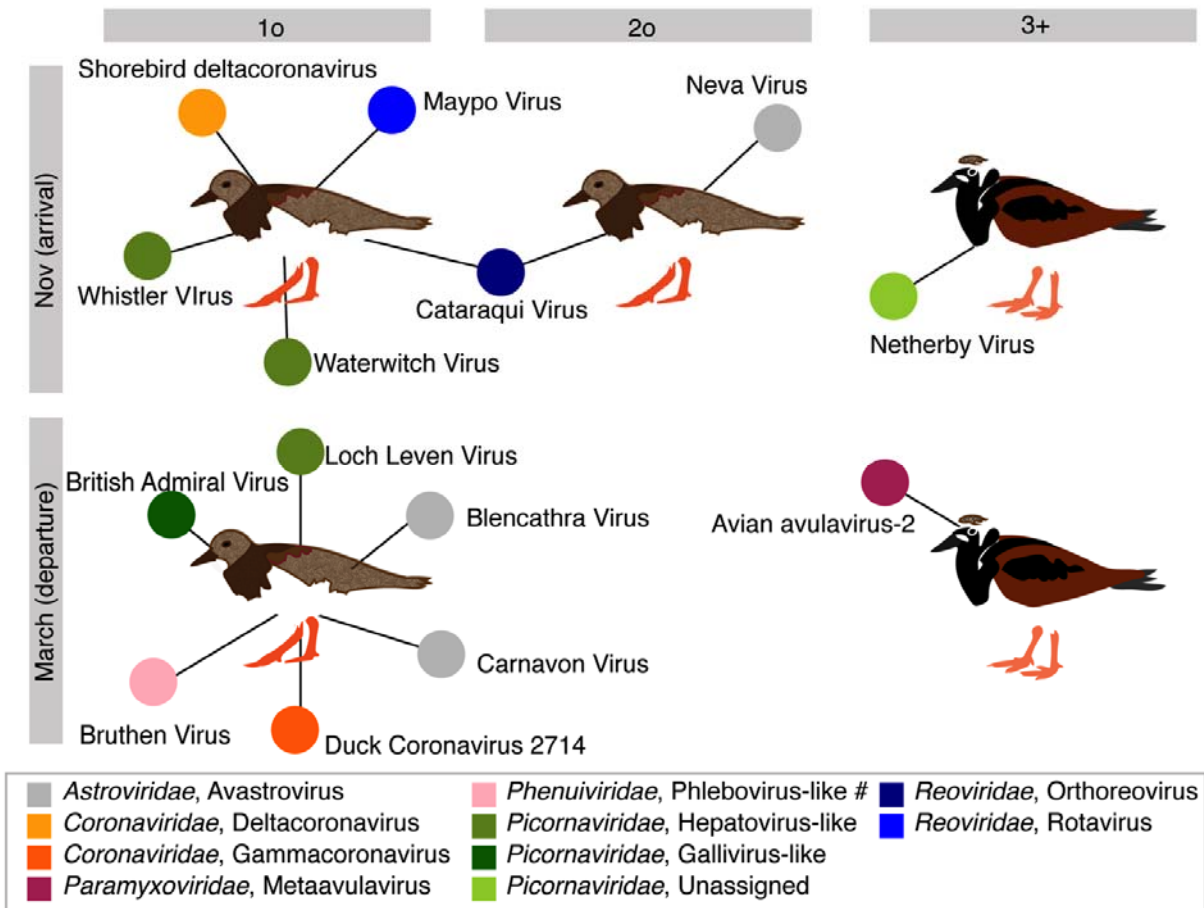
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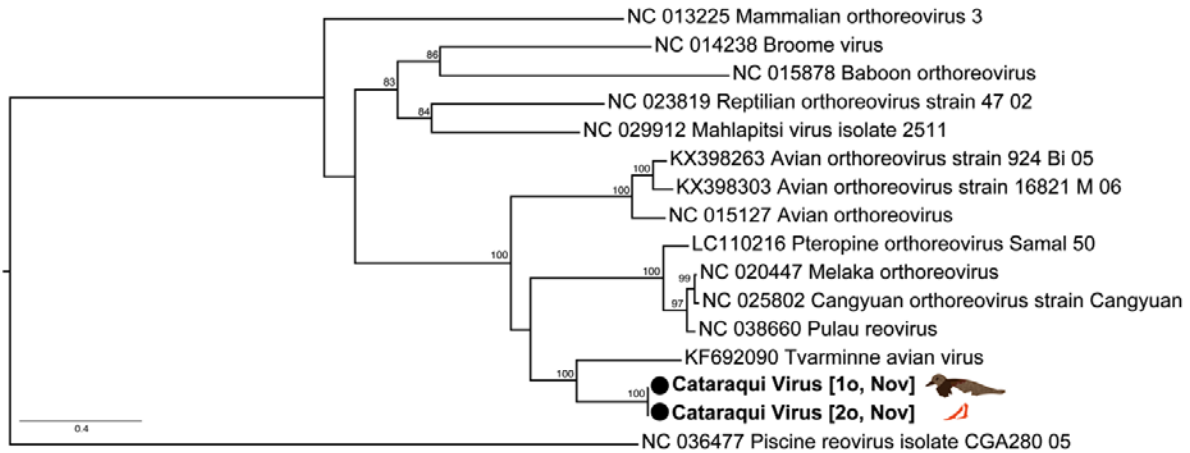
521 **Figure 1. Sampling scheme.** Birds occupied three age categories: juvenile (1o) birds were  
522 individuals hatched in Siberia that had performed a single migration leg to King Island; adolescent  
523 (2o) (that could only be identified based on their plumage in November) were individuals who had  
524 overwintered in Australia; and adult (3+) birds that perform an annual migration to Siberia and back  
525 to breed each year. Ruddy Turnstones arrive on King Island in October and can be found on rocky  
526 shores of the western coastline of the island throughout the Australian summer period. Small groups  
527 of ~50 birds with high site fidelity occupy beaches, with surveyed beaches denoted in small white  
528 circles. In autumn, following a period of intense weight gain, adult birds depart on migration to  
529 their breeding areas in March/April. Samples used in this study were collected in November 2014  
530 and March 2015.



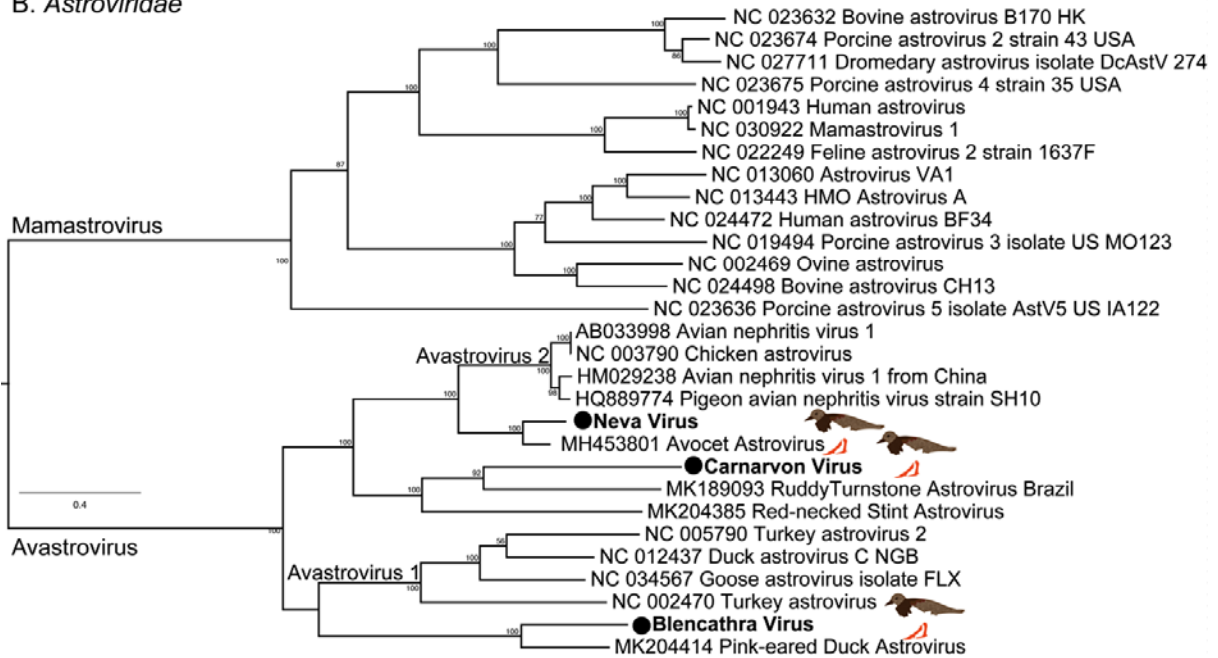
531

532 **Figure 2.** Bipartite network illustrating species for which complete viral genomes were found in  
533 each library, comprising 10 individuals. Each library is represented as a central node, with a  
534 pictogram of the respective age cohort of Ruddy Turnstone, surrounded by each viral species.  
535 Where two libraries share a virus species, the networks between the two libraries are linked.  
536 Placement of libraries is arranged by sampling date on the y-axis and age on the x-axis. Virus  
537 colour corresponds to virus taxonomy. A list of viruses from each library is presented in Table S2,  
538 and phylogenetic trees for each virus family and species can be found in Fig 3–5, Fig S2–S7.

A. *Reoviridae*, Orthoreovirus

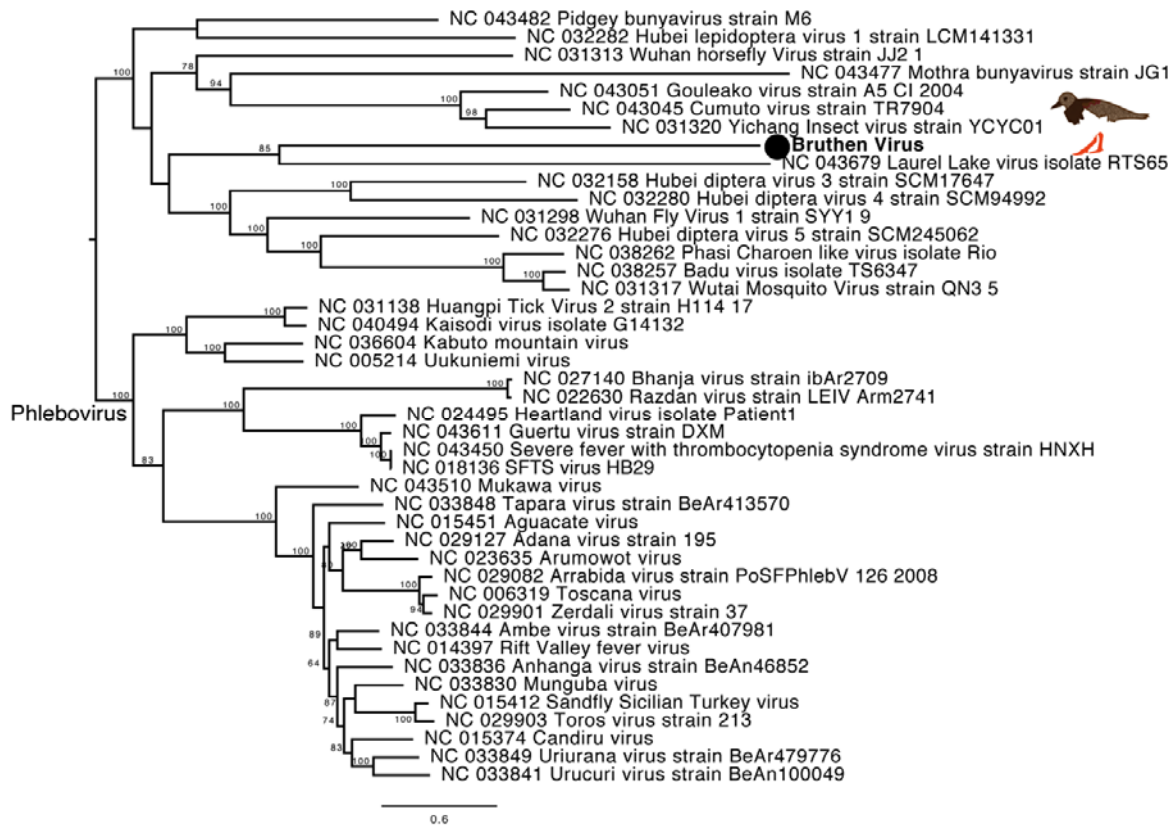


B. *Astroviridae*



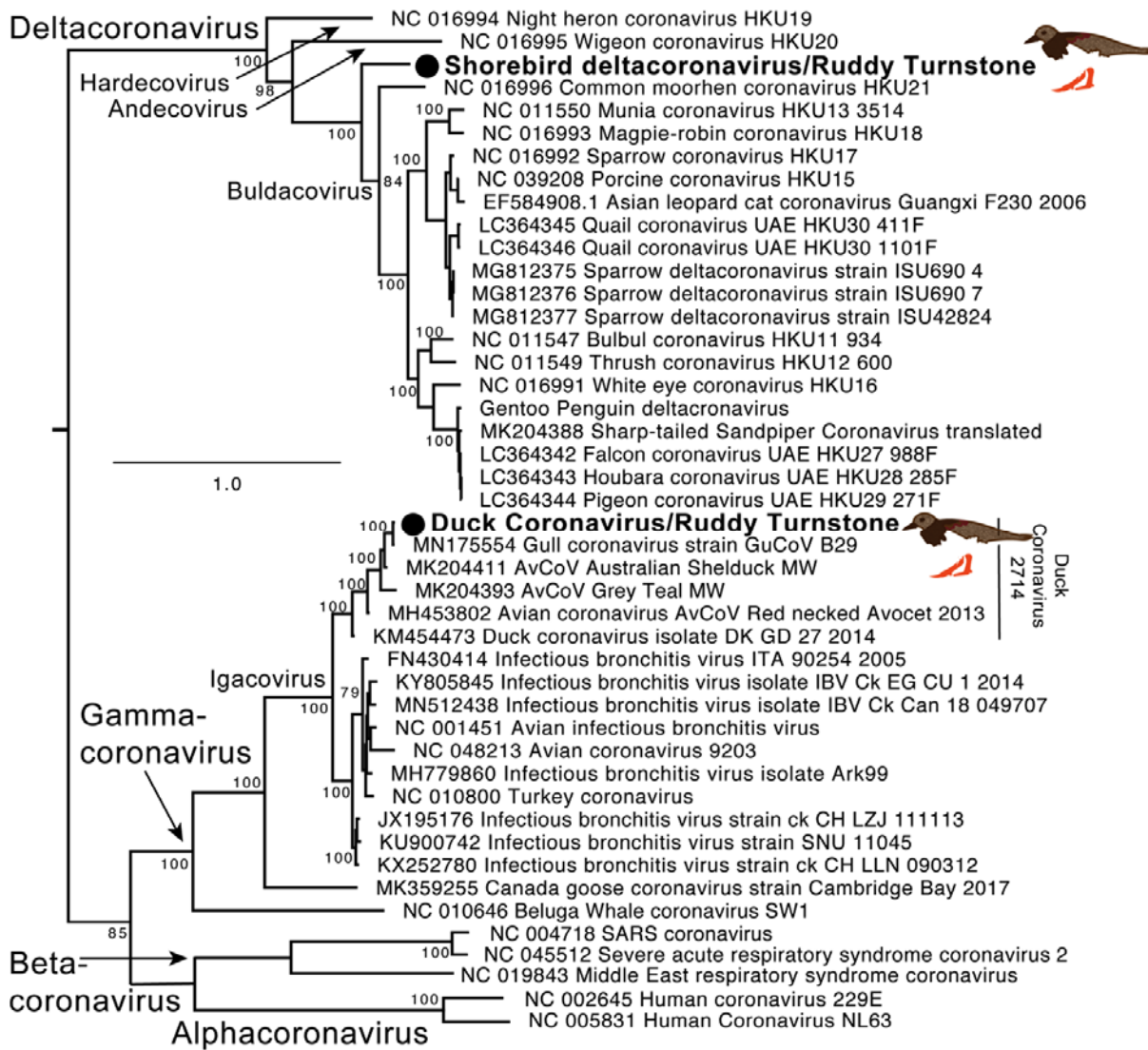
539

540 **Figure 3.** (A) Phylogenetic tree of the LambdaB segment, containing the RdRp, of orthoreoviruses.  
 541 Piscine reovirus is set as the outgroup. (B) Phylogenetic tree of the ORF1ab, including the RdRp, of  
 542 avastroviruses. Avastrovirus 3 is not shown as there are no full genomes available, but is presented  
 543 in Fig S2 which shows a phylogeny inferred from a short region of the RdRp. The tree was  
 544 midpoint rooted, corresponding to division between mammalian and avian viruses. The sequences  
 545 generated in this study are indicated by a filled circle and are in bold. Bootstrap values >70% are  
 546 shown for key nodes. The scale bar indicates the number of amino acid substitutions per site.



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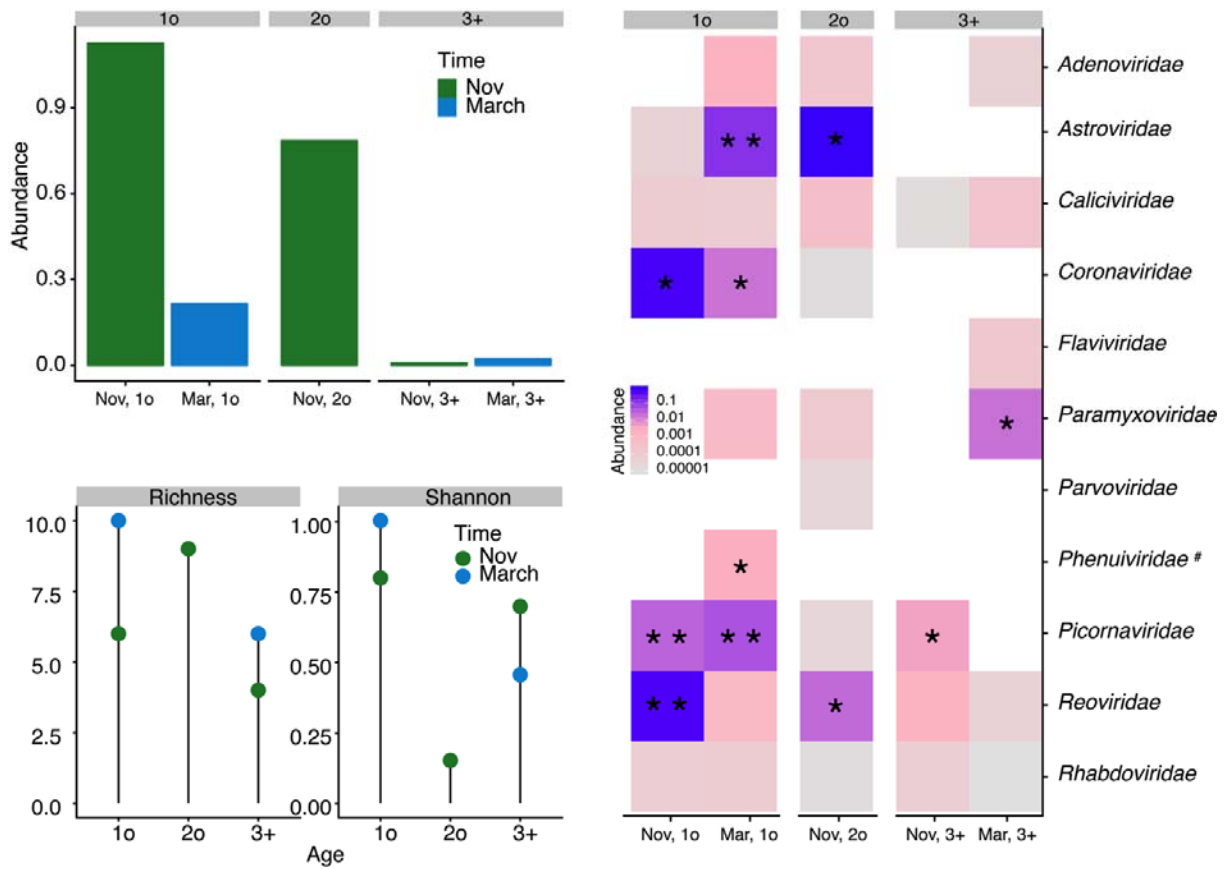
548 **Figure 4.** Phylogeny of the polyprotein of the *Phenuiviridae*. The tree was midpoint rooted for  
549 clarity only. Viruses described in this study are marked in bold, adjacent to a filled circle. Bootstrap  
550 values >70% are shown for key nodes. The scale bar indicates the number of amino acid  
551 substitutions per site.



552

553 **Figure 5.** Phylogenetic tree of the ORF1ab, containing the RdRp, of the *Coronaviridae*. The  
 554 sequences generated in this study are indicated by a filled circle and are in bold. Deltacoronaviruses  
 555 are set as the outgroup, as per [42]. Only select members of the alpha- and betacoronaviruses are  
 556 included. Bootstrap values >70% are shown for key nodes. The scale bar indicates the number of  
 557 amino acid substitutions per site. Partial gammacoronavirus and deltacoronavirus phylogenies,  
 558 including numerous wild bird sequences, are presented in Fig S5 and S6, respectively.





559

560 **Figure 6** Effect of age on abundance and alpha diversity. (A) Viral abundance is higher in 1o birds  
 561 compared to 3+ birds. (B) Alpha diversity at the viral family level, here measured as observed  
 562 richness and Shannon diversity, is higher in 1o birds compared to 3+ birds. Alpha diversity at the  
 563 virus genus level is presented in Fig S1. (C) Heatmap illustrating viral diversity, at the genus level  
 564 in each library, with colour corresponding to viral abundance. Blue and purple correspond to viruses  
 565 with high abundance, and pink corresponds to viruses with low abundance. Asterisks indicate cases  
 566 in which at least one complete viral genome was obtained. Viral families marked with a hash  
 567 symbol indicate families where host association is unclear. Plots for the virus genus level are  
 568 presented in Fig S8.