

# Detection of H3N8 influenza A virus with multiple mammalian-adaptive mutations in a rescued Grey seal (*Halichoerus grypus*) pup

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## Abstract

Avian influenza A viruses (IAVs) in different species of seals display a spectrum of pathogenicity, from sub-clinical infection to mass mortality events. Here we present an investigation of avian IAV infection in a 3- to 4-month-old Grey seal (*Halichoerus grypus*) pup, rescued from St Michael's Mount, Cornwall in 2017. The pup underwent medical treatment but died after two weeks; post-mortem examination and histology indicated sepsis as the cause of death. IAV NP antigen was detected by immunohistochemistry in the nasal mucosa, and sensitive real-time reverse transcription polymerase chain reaction assays detected trace amounts of viral RNA within the lower respiratory tract, suggesting that the infection may have been cleared naturally. IAV prevalence among Grey seals may therefore be underestimated. Moreover, contact with humans during the rescue raised concerns about potential zoonotic risk. Nucleotide sequencing revealed the virus to be of subtype H3N8. Combining a GISAID database BLAST search and time-scaled phylogenetic analyses, we inferred that the seal virus originated from an unsampled, locally circulating (in Northern Europe) viruses, likely from wild Anseriformes. From examining the protein alignments, we found several residue changes in the seal virus that did not occur in the bird viruses, including D701N in the PB2 segment, a rare mutation, and a hallmark of mammalian adaptation of bird viruses.

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IAVs of H3N8 subtype have been noted for their particular ability to cross the species barrier and cause productive infections, including historical records suggesting that they may have caused the 1889 pandemic. Therefore, infections such as the one we report here may be of interest to pandemic surveillance and risk and help us better understand the determinants and drivers of mammalian adaptation in influenza.

**Key words:** influenza; grey seal; avian influenza; viral reservoir; pinniped; mammalian adaptation.

## 1. Introduction

Influenza A viruses (IAVs) are important pathogens for humans and livestock including pigs and poultry. They are segmented RNA viruses, whose genomes consist of eight segments of RNA, which code for ~11 proteins/polypeptides. IAVs are classified into several subtypes based on the antigenic properties of two surface glycoproteins haemagglutinin (HA, avian subtypes H1–H16) and neuraminidase (NA, avian subtypes N1–N9). Viruses of most subtypes can be found in wild waterfowl and shorebirds which are their natural reservoir (Easterday et al. 1968; Alexander 2007) and can infect both domestic birds and mammalian species in spill-over infections. A few IAV lineages have become established in mammals such as humans, pigs, horses, and dogs though only of subtypes H1, H2, and H3 in combination with N1, N2, or N8 (Webster et al. 1992; Reperant, Rimmelzwaan, and Kuiken 2009).

IAV in birds replicates mainly in the intestine and is transmitted through the faecal-oral route, although respiratory tropism and oropharyngeal shedding has been noted (Webster et al. 1978; Daoust et al. 2011, 2013; França et al. 2012; Höfle et al. 2012), and the virus can survive in the environment for fairly long periods (Stallknecht et al. 1990; Brown et al. 2009). This creates conditions conducive to viral exchange with marine mammals such as pinnipeds (seals), whose habitats and prey intersect with those of waterfowl and shorebirds. Several cases of marine mammal infection with IAV of many subtypes including H1N1, H3N3, H3N8, H4N5, H4N6, H7N7, and H10N7 have been documented with a spectrum of effects ranging from mass die-offs to sub-clinical; in a majority of these cases, the source is implicated to be avian (White 2013; Fereidouni et al. 2016). A few studies have also suggested that Grey seals may act as an endemically infected reservoir which may disseminate viruses in coastal ecosystems to other mammals, coastal birds, and potentially humans (Duignan et al. 1995, 1997; Puryear et al. 2016). Indeed, seroprevalence levels in live-captured healthy Grey seal populations (20–26%) (Bodewes et al. 2015; Puryear et al. 2016) is comparable with levels found in wild birds (31–60%) depending on species, geography, seasonality, and other factors (Fereidouni et al. 2010; Wilson et al. 2013; Curran, Ellis, and Robertson 2015).

Seals may also be infected by IAV from non-avian sources; there is serological evidence for infection of Baikal and ringed seals in Russia with human H3N2 strains A/Aichi/2/68 and A/Bangkok/1/79 (Ohishi et al. 2004) and a human pandemic-2009 H1N1 virus was isolated from Elephant seals in 2010 on the coast of California, USA. Furthermore, it has been shown that IAV from seals can replicate in human tissue and that seal IAV can be systemically virulent in primates (Webster et al. 1981; Murphy et al. 1983; White 2013). Seals may therefore be potential sources of pandemic influenza.

In this article, we report an H3N8 IAV infection of a rescued Grey seal pup in coastal England. We provide molecular and histological evidence for presence of IAV in the seal's respiratory tissues and conclude from the post-mortem that the clinical

presentation was not caused by IAV. To our knowledge, we provide the first whole-genome sequence for an IAV isolated from a Grey seal, as attempts so far have been unsuccessful. We use phylogenetic analyses to find the putative sources of this virus and look for adaptive changes in its sequence. We compare this case with previously described seal infections, particularly the other H3N8 virus A/harbor\_seal/Massachusetts/1/2011, to identify unique or parallel elements which may have implications for animal and human health.

## 2. Methods

### 2.1 Clinical history and medical interventions

In February 2017, a female Grey seal (*Halichoerus grypus*) pup was rescued from St Michael's Mount, Cornwall. Physical examination of the animal on admittance to the rehabilitation centre revealed an emaciated and dehydrated subject (body weight = 20 kg). The estimated age was 3–4 months. The animal exhibited a mucoid nasal discharge and a 1 cm wound on the ventral thorax. Its temperature was 39.9°C and breathing rate was abnormal (continuous breathing pattern, 12 breaths per minute). The pup was monitored and provided nutritional support, fluid therapy, and antibiotic treatment, but died suddenly 14 days after admittance. The carcass and a nasal swab were submitted to the Animal and Plant Health Agency (APHA) for virological investigation.

### 2.2 Pathology, histopathology, and immunohistochemistry

The carcass underwent a full post-mortem examination. A set of tissues was sampled and fixed in buffered formalin (nasal turbinates, trachea, lung, kidney, soft tissues adjacent to the cutaneous/subcutaneous lesion, and lymph node). A standard histopathological examination was carried out on the tissues (haematoxylin & eosin), followed by an immunohistochemical investigation targeting nucleoprotein to detect intra-lesional IAV along the respiratory tract (Brookes et al. 2010).

### 2.3 Real-time reverse transcription polymerase chain reaction

RNA was extracted from the nasal swab and tissue suspensions using the QIAmp viral RNA BioRobot kit customised for APHA in conjunction with a Universal BioRobot (Qiagen, Manchester, UK) (Slomka et al. 2009). Real-time reverse transcription polymerase chain reaction (RRT-PCR) testing of the RNA extracts comprised (1) the Matrix (M)-gene assay for generic IAV detection using the primers and probes of (Nagy et al. 2010) and (2) H5 and H7 IAV RRT-PCR assays to test for notifiable avian influenza (Slomka et al. 2007, 2009). For each RRT-PCR assay, samples producing a threshold cycle (CT) value <36.0 were considered positive (Slomka et al. 2010). The RNA was also tested by an IAV N1-specific RRT-PCR according to the procedure described by Payungporn et al. (2006), an IAV N5-specific RRT-PCR

(James et al. 2018) and two IAV subtype N8-specific RRT-PCRs (James et al. 2018) with the same positive/negative acceptance criteria. All amplifications were carried out in an MX3000P qPCR System (Agilent).

## 2.4 Viral whole-genome sequencing

Attempted virus isolation in 9- to 11-day-old SPF embryonated fowls' eggs was performed on the nasal swab sample as well as tissue samples according to the internationally recognised European Union (EU) and OIE methods (EU 2006; OIE 2015), but was unsuccessful. RNA was sequenced using the MiSeq platform. Briefly, viral RNA was extracted from the nasal swab using the QIAmp viral RNA mini-kit without the addition of carrier RNA (Qiagen, Manchester, UK). cDNA was synthesised from RNA using a random hexamer primer mix and cDNA Synthesis System (Roche, UK). The Sequence library was prepared using a NexteraXT kit (Illumina, Cambridge, UK). Quality control and quantification of the cDNA and Sequence Library was performed using Quantifluor dsDNA System (Promega, UK). Sequence libraries were run on a MiSeq using MiSeq V2 300 cycle kit (Illumina, Cambridge, UK) with  $2 \times 150$  base paired-end reads. The raw sequence reads were analysed using publicly available bioinformatics software, following an in-house pipeline, available on github (<https://github.com/ellisrichardj/FluSeqID/blob/master/FluSeqID.sh>). This pipeline de novo assembles the raw data using the Velvet assembler (Zerbino and Birney 2008), Basic Local Alignment Search Tools (BLASTs) the resulting contigs against a local database of influenza genes using Blast+ (Camacho et al. 2009), then maps the raw data against the highest scoring blast hit using the Burrows-Wheeler Aligner (Li 2013). The consensus sequence was extracted from the resultant bam file using a modified SAMtools software package (Li et al. 2009), script (vcf2consensus.pl) available at: [https://github.com/ellisrichardj/csu\\_scripts/blob/master/vcf2consensus.pl](https://github.com/ellisrichardj/csu_scripts/blob/master/vcf2consensus.pl). Whole-genome sequence of the virus is available on GISAID database with unique ID: EPI\_ISL\_381748.

## 2.5 BLAST and phylogenetic analysis of the internal genes of all available seal viruses

We downloaded all available sequences for each internal gene segment (with duplicates removed) from the influenza research database website ([www.fludb.org](http://www.fludb.org)) from which a blast database was created locally. It was not feasible to download sequences in bulk from GISAID. Seal internal gene segment sequences of all twelve seal viruses with available whole-genome sequences to be used as query were downloaded from the GISAID (Elbe and Buckland-Merrett, 2017; Shu and McCauley, 2017). BLAST was run locally with the number of returnable alignments restricted to fifteen per query. Sequences of BLAST hits were extracted and split into segment-wise files and respective query sequences were added to each file. For each segment dataset sequences duplicate strain names were discarded and aligned using MAFFT (Katoh and Standley 2013) using automatic settings. Alignments for each segment were inspected manually on AliView (Larsson 2014) and the ends trimmed to the starting ATG and end STOP codon. Trees were run using FastTree (Price, Dehal, and Arkin 2009), and plotted in R v3.6 using the ggtree package (Yu et al. 2017). Tree files and the R scripts used to plot them are available online at: [https://github.com/delfinut/seal\\_influenza\\_ms.git](https://github.com/delfinut/seal_influenza_ms.git).

## 2.6 BLAST and whole-genome time-scaled phylogenetic analysis of H3N8 seal viruses

BLAST was used on GISAID (Elbe and Buckland-Merrett 2017; Shu and McCauley 2017) to find the closest-related viral segments for each segment of the grey seal virus. After checking that >200 BLAST hits were all largely avian, we limited the search to the top 50 hits for further analyses. We combined the seal virus sequence (query) along with the BLAST hits sequences (blasthit) for each segment for phylogenetic analysis. We removed sequences containing duplicate strain names and aligned with MAFFT (Katoh and Standley 2013) using automatic settings. Alignments for each segment were inspected manually on AliView (Larsson 2014) and the ends trimmed to the starting ATG and end STOP codon. Trees were first run using FastTree (Price, Dehal, and Arkin 2009), after which we used IQ-TREE (Nguyen et al. 2015) to make the final maximum-likelihood tree with 1000 iterations of alrt (approximate likelihood ratio test) for branch support. Tempest v1.5 (Rambaut et al. 2016) was used to test ML trees for clock-like behaviour. Trees for all segments except MP showed clock-like behaviour (Supplementary Fig. S1A), so results from the MP dataset were excluded. BEAST v1.10.1 (Bayesian Evolutionary Analysis Sampling Trees) (Suchard et al. 2018) was used to determine the putative time and source of emergence of the different segments of the seal virus. BEAST performs Bayesian analysis of molecular sequences using Markov chain Monte Carlo (MCMC) methods. For all segments other than MP and NS which have multiple reading frames, we used the SRD06 site model which partitions the codons into 1 + 2 and 3. For NS we used a GTR model with no codon partitioning. The tree prior was kept identical for all segments: Gaussian Markov random field Bayesian skyride tree (Minin, Bloomquist, and Suchard 2008). We tested three clock models: strict, uncorrelated relaxed (lognormal distribution), and random local using path sampling (PS)/stepping stone sampling (SS) marginal-likelihood estimation calculations (1 million chains, with likelihood logged every 800, with 80PS/SS steps) and found that in all cases the random local clock was preferred with log Bayes factors ranging from 0.4 to 6.2 with the next best model (summarised in Supplementary Table S1A). Three separate runs of 80 million MCMC generations with parameters logged every 8000 were performed for each segment, which were combined after logs were inspected in Tracer v1.7.1 (Rambaut et al. 2018) for appropriate mixing and effective sample size values > 200. Trees were summarised into median clade credibility (MCC) trees and plotted in R v3.6 using the ggtree package (Yu et al. 2017). As a comparison, the same analysis from BLAST to BEAST was performed for all segments of the 2011 H3N8 virus A/harbor\_seal/Massachusetts/1/2011. In this case, the log Bayes factor values ranged between 0.08 and 7.9 with the next best model (also summarised in Supplementary Table S1B). Four separate runs were needed for convergence of PB1 segment dataset, while three runs were sufficient for all other segments. All runs were consistent in their parameter estimations (as inferred by comparison by eye in Tracer). BEAST xml files, MCC tree files, and R scripts used to plot the trees are available online at: [https://github.com/delfinut/seal\\_influenza\\_ms.git](https://github.com/delfinut/seal_influenza_ms.git).

## 2.7 Amino acid substitutions

Trimmed alignments of each segment were manually inspected in AliView software (Larsson 2014), translated into amino acids, and checked for amino acid changes across each dataset.

We identified several substitutions in the seal virus that did not occur in any of the bird virus sequences. We recorded these substitutions, and used the H3 numbering of the sequence using the HA subtype numbering conversion tool available from FluDB (Burke and Smith 2014, <https://tinyurl.com/HAnumbering>) for the HA protein. We also looked for differences in glycosylation patterns between the seal and the related wild bird HA and NA glycoproteins using a programme to detect Asn-X-Ser or Asn-X-Thr (where X is any amino acid other than proline) patterns in the amino acid sequences (Todd Davis, pers. comm., CDC, USA).

### 3. Results

#### 3.1 Pathology and immunohistochemistry

The post-mortem revealed abundant purulent fluid (approximately 1l) in the subcutaneous tissues of the right side of the body extending from the neck to the thoracic region (Fig. 1A). All other organs were macroscopically unremarkable. The blubber sternal thickness was 0.4 cm. Histology identified a severe, chronic-active, fibrino-purulent cellulitis and fasciitis and a severe, acute renal infarct with thrombosis. In the respiratory system, a diffuse mild to moderate rhinitis with epithelial hyperplasia and presence of mites was observed in the nasal cavity and a focal broncho-interstitial pneumonia with thrombosis and pulmonary nematodes were seen in the lungs, with no changes observed in trachea and bronchi. IAV antigen was detected by immunohistochemistry (IHC) only in the nasal mucosa, in the nuclei of scattered isolated epithelial cells (Fig. 1B). The pathological findings were suggestive of a thromboembolic event and sepsis caused by the cellulitis as the cause of the death of the animal.

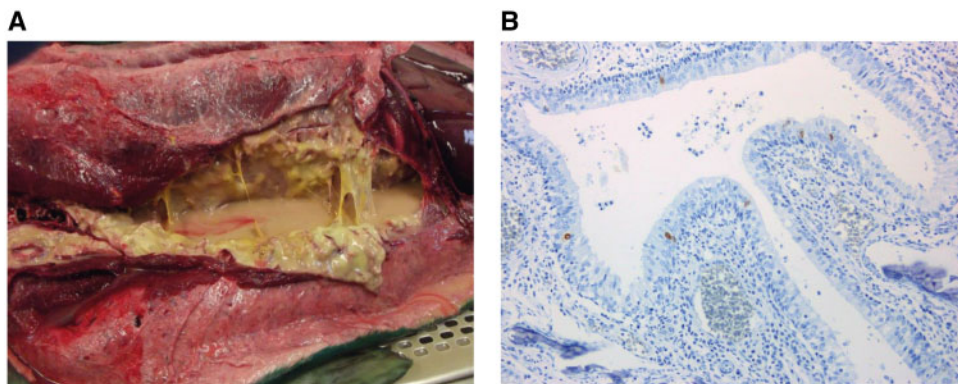
#### 3.2 Detection and subtyping of IAV by RRT-PCR

RRT-PCR testing of the RNA extracts from the nasal swab was positive for the M-gene (CT value of 22.36), signifying the presence of IAV RNA but was negative for notifiable avian influenza HA subtypes H5 and H7 as well as the IAV subtypes N1 and N5. However, IAV subtype N8 was detected by one of the specific RRT-PCR assays employed (CT value of 24.38). In addition, trace amounts of IAV RNA were detected by the M-gene RRT-PCR assay in tracheal bronchi (CT value of 35.88), the left lung (CT value of 37.73), and the right cranial lung (CT value of 38.67). Due to low levels of virus detected in the lower respiratory tract,

and the presence of clinical signs not consistent with influenza infection, we concluded that the virus was of no or low virulence. Sequencing results from the nasal swab indicated no mixed infection, just a single virus, from which it was possible to sequence all eight gene segments. The virus-derived sequence was named A/Grey seal/England/027661/2017.

#### 3.3 Source of viral segments

We used BLAST against the GISAID database to find viral strains whose sequences were most similar to A/Grey seal/England/027661/2017. The 50 closest-matching sequences were overwhelmingly of avian influenza virus origin, but one human and a few sequences from other seal IAV previously isolated in Europe were also present. The strains in the set of BLAST hits for each segment gene, all came from different avian influenza viruses, isolated from different bird types, and of different viral subtypes. They were from strains that were mostly isolated from wild birds such as mallard, Eurasian teal, White-fronted goose, Black-headed gull (*Anas platyrhynchos*, *Anas crecca*, *Anser albifrons*, *Chroicocephalus ridibundus*), and others, along with a few from domestic birds such as chickens (*Gallus gallus domesticus*). Similar to previously reported infections (Anthony et al. 2012; Zohari et al. 2014), the set of closest-related strains were isolated from within the local region, in this case, Northern Europe (The Netherlands, Germany, UK, etc.) with the exception of three strains from China being among the BLAST hits of the PB1 sequence. The years of detection of the closest-related avian IAV for each segment ranged from the 2007 (NA) to 2015 (NP/PB1/PB2). We performed a similar BLAST analysis for the A/harbor\_seal/Massachusetts/1/2011 virus and found similar patterns in that the 50 closest-matching sequences were avian in origin, of multiple subtypes and isolated from different bird species; however, no human or other seal IAV strains were found in this case. A/blue-winged teal/Ohio/926/2002(H3N8) was previously reported to be the closest-related avian IAV (Anthony et al. 2012), however, our BLAST results find avian viruses more recently isolated and a more restricted time frame (2009–11). It is possible that the authors missed these viruses as they might not have been publicly available at the time of the analysis in 2012. It is also possible that the search was restricted to H3N8 viruses, which given our recent understanding of the high levels of reassortment in avian IAV across hosts and subtypes (Wille et al. 2013; Lu, Lycett, and Leigh Brown 2014; Venkatesh et al. 2018) would be inappropriate.



**Figure 1.** (A) Grey seal pup post-mortem. Gross post-mortem examination of the subcutaneous tissues and skeletal muscle over the thorax revealed a focally extensive, chronic-active, purulent, and fibrino-necrotising cellulitis. This lesion was considered predisposing factors for a fatal sepsis as ultimate cause of death of the pup. (B) IHC of Grey seal pup nasal mucosa. Brown labelling (DAB chromogen) indicates the presence of viral antigen (influenza nucleoprotein). There are no histological signs of viral driven tissue damage. (DAB chromogen and haematoxylin counterstain) (original magnification: 400×).

**Supplementary Table S2A** shows a summary for how often the same strain appears as a BLAST hit for each segment of the seal virus A/Grey seal/England/027661/2017. A large proportion of these occur singly (250 strains) or for a maximum of two segments (40 strains). However, some strains were found to map for 3, 4, or 5 segments (19, 2, and 1 strains, respectively). Of these, all the strains that mapped for at least three segments were isolated from birds in the Netherlands between 2011 and 2015, except for one chicken virus from France in 2016. The subtypes varied greatly, but they were usually isolated from wild birds. **Supplementary Table S2B** shows a summary for how often the same strain appears as a BLAST hit for each segment of the seal virus A/harbor\_seal/Massachusetts/1/2011. Again, a large proportion occur singly (319 strains), or a maximum of two segments (21 strains), while two strains had three segments each. In contrast to A/Grey seal/England/027661/2017, however, three viruses isolated within a day of each other from American black ducks in New Brunswick in 2009 were found to appear among the BLAST hits for all six internal genes.

There were no common BLAST hits between the A/harbor\_seal/Massachusetts/1/2011 and A/Grey seal/England/027661/2017 so we can infer that these seal viruses are epidemiologically unlinked and derive from different pools of avian IAV. We did a phylogenetic analysis of the BLAST hits of the internal genes of all available whole-genome seal viruses to date (see **Fig. 2** and **Supplementary Fig. S5**). These include twelve seal origin influenza viruses from several seal species, which are derived from avian and human sources. **Figure 2** shows that for each seal virus, the PB2 segment sequence largely clusters with its own set of similar avian or human influenza viruses—this is largely true for other segments as well (**Supplementary Fig. S5**). In the figures, seal IAV are shown in blue, and BLAST hits that occur only for one segment are shown in grey. BLAST hits for two or more segments are in progressively darker shades of pink. We see that the case of A/harbor\_seal/Massachusetts/1/2011, with all internal genes potentially derived from the same wild bird virus, is an outlier. It is more common to have higher levels of reassortment, i.e. fewer segments (2–4) from each source virus.

In the next section, we further examine the closest-available sequence(s) for each Grey seal gene segment to try and understand its emergence.

### 3.4 Emergence of viral segments

We performed a time-scaled analysis with the set of closely related avian virus segment sequences, to test if all segments from seal virus had a similar point of introduction, i.e. similar time to most recent common ancestor (TMRCA) of the seal and wild bird influenza viruses for all segments, or the extent to which this varied segment to segment. We used BEAST to reconstruct time-scaled phylogenies for each of our segment datasets for the Grey seal virus.

The maximum clade credibility (MCC) trees for HA, NA, and PB2 segments are presented in **Fig. 3A**; while those for all segments except MP are provided in **Supplementary Fig. S2**. A maximum-likelihood tree for MP is shown in **Supplementary Fig. S3**. We do not show the BEAST analysis for the MP dataset as it lacked a temporal signal when analysed by Tempest v1.5 (**Supplementary Fig. S1A**), which is necessary for adequate inference of TMRCA. Time to the putative ancestor strain of the segments from the seal virus and its closest-related segment is inferred as ranging from 1999 (NA) to between 2011 and 2015 (all other gene segments), summarised in **Table 1A**. Gaps in

surveillance, and the availability of just one seal strain will likely affect the inference of TMRCA, but the variation between segments is also likely a testament to the high levels of reassortment seen in wild bird IAVs (Wille et al. 2013; Lu, Lycett, and Leigh Brown 2014; Venkatesh et al. 2018). Indeed, the closest-associated virus varies in host, subtype, and geography of isolation for each segment, as can be seen in the highlighted clades in **Fig. 3A–C** and **Supplementary Fig. S2**. Consistent with data of BLAST hits (**Supplementary Table S1**), many of the closest-related segments are from strains isolated in the Netherlands (HA, MP, NP, NS, PA, PB1, and PB2). Where multiple strains are equally closely related to the seal virus, e.g. for PB1 and PB2, the avian influenza virus strains come from the Netherlands and France. The closest strain to the seal NA gene that has been sampled is from 2009 at the latest, from Norway.

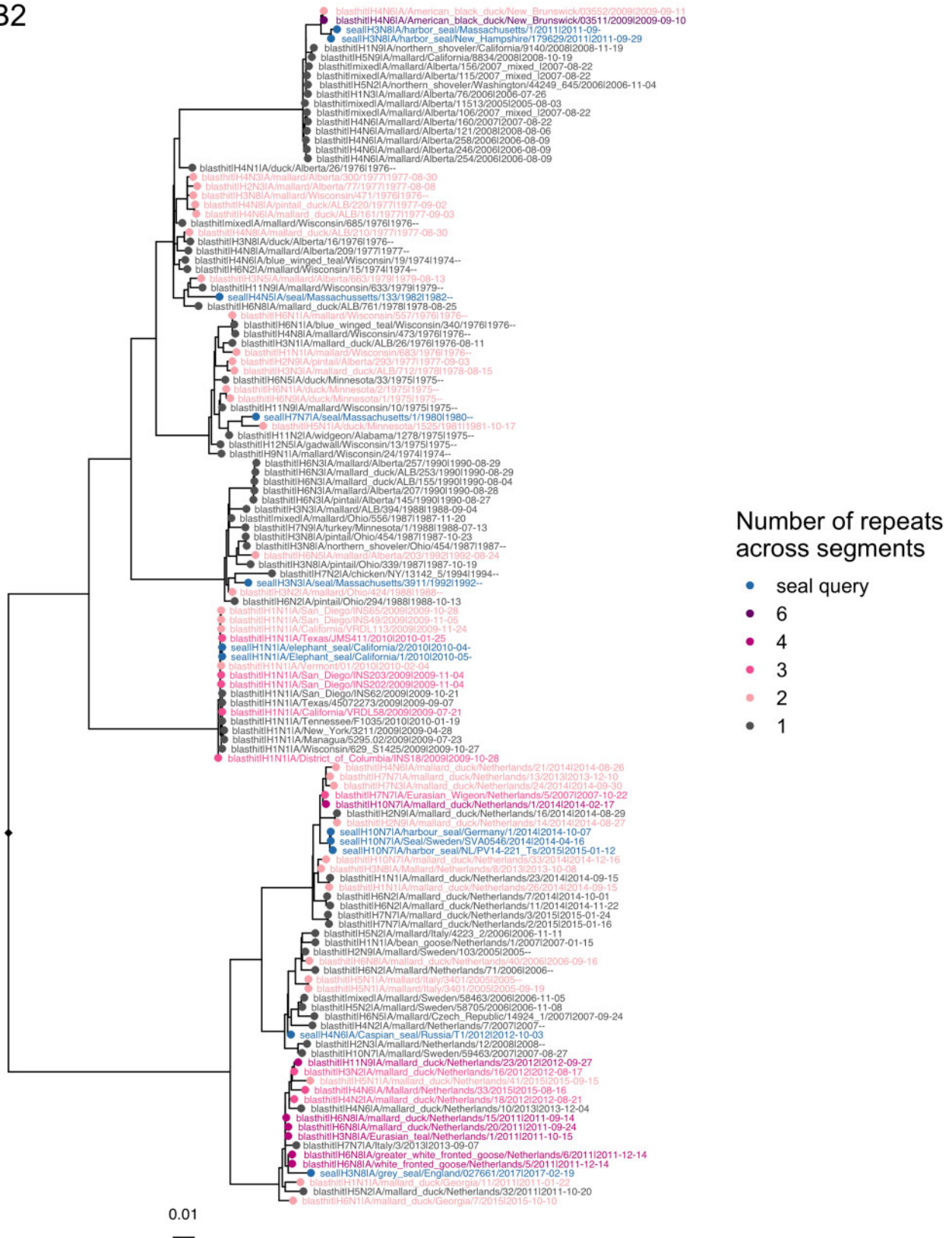
The closest-related strains to the A/harbor\_seal/Massachusetts/1/2011 virus segments are much more restricted in their origin. For all internal gene segments as identified by BLAST and maximum-likelihood trees, they are a set of H4N6 viruses from American black ducks in New Brunswick, isolated on 10 or 11 September 2009. See **Supplementary Fig. S4A–H**. The closest-related strains for the glycoproteins are different, indicating reassortment events or possibly, bias for sequencing glycoproteins over the internal genes. The MCC trees for all segments are provided in **Supplementary Fig. S4A–H**. The closest-related HA sequence is not resolved as the seal HA forms an external branch to avian influenza viruses isolated from Blue-winged Teals from New Brunswick in 2010 and mallards from Ohio in 2011. The seal NA is also similarly grouped with viruses isolated from mallards/Northern pintail in Minnesota in 2009. While posterior support values for these nodes are not high (see **Table 1B**), support one node deeper is very high (1.0). Despite at least three different avian sources for the Harbour seal virus segments, they show a restricted TMRCA to putative ancestor strains. **Table 1B** shows that the mean TMRCA for different segments ranges from the end of 2007 (2007.9, HA) to late 2009 (2009.97, MP)—approximately two years. In comparison, the Grey seal origin viral segments range between ~1999 and ~2015–18 years, if NA is included, or at least 4 years (~2011–15) for rest of the segments.

### 3.5 Substitutions for mammalian adaptation

Previous analyses have indicated that mammalian-adaptive mutations can occur in avian influenza viruses when they are transmitted into seals. A study of H10N7 viruses in Harbour seals in Northern Europe, which unlike the present case was demonstrated to have transmitted to other seals and caused an outbreak, showed that mutations were likely to occur early on after transmission to seals and then plateau (Bodewes et al. 2016). We looked for differences between the seal virus and wild bird IAVs in the segment amino acid alignments of our datasets, to check if they any had putative adaptive implications.

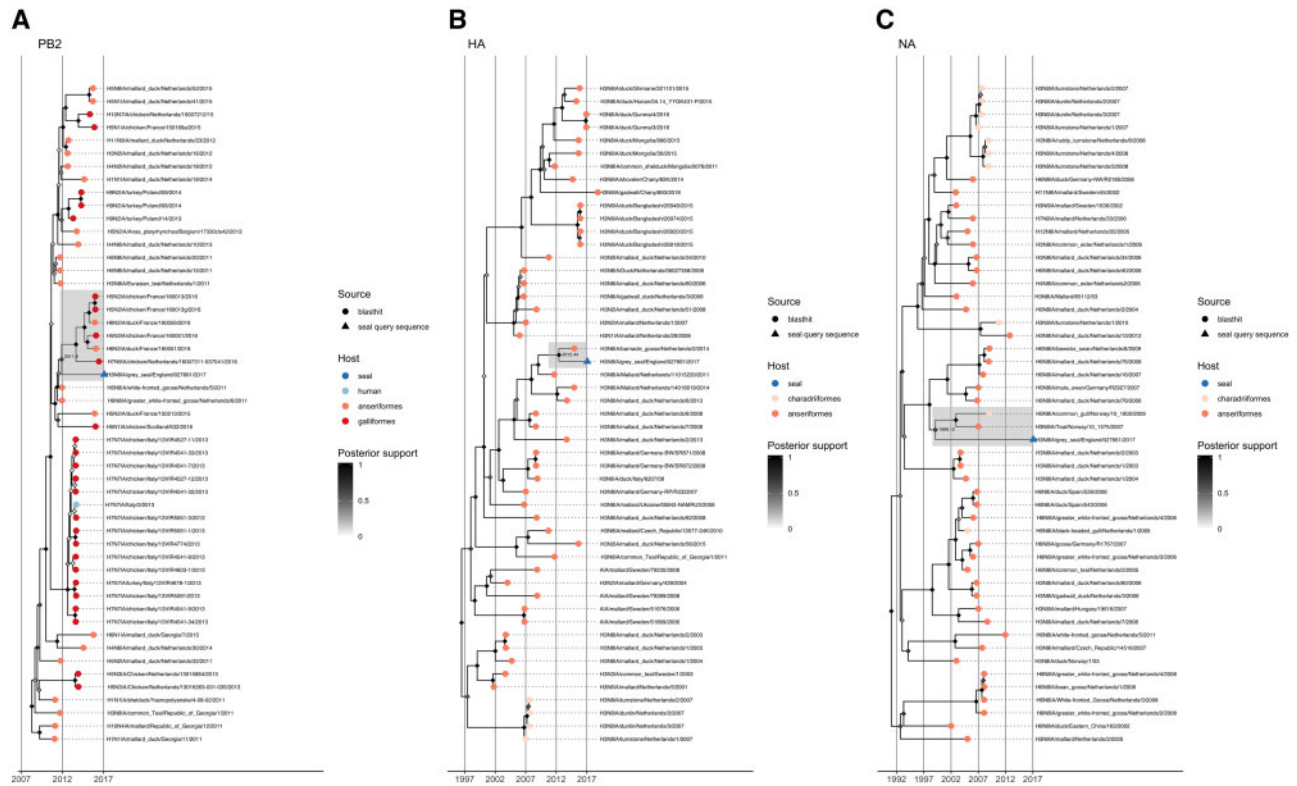
We found up to 22 amino acid substitutions in the seal influenza virus that did not occur in any of the related bird viruses. These substitutions are summarised in **Table 2**, along with references for those that have been identified in previous studies of mammalian adaptation (previously identified substitutions are indicated in bold font). Many of these changes occur in the polymerase complex genes (Mänz, Schwemmler, and Brunotte 2013): D701N in the PB2 segment is a rare mutation, and a hallmark of mammalian adaptation of bird influenza viruses, regardless of genetic background (Steel et al. 2009; Liu et al. 2018). Liu and Steel et al. have elucidated that the basis of this adaptation is that it allows for better replication in

PB2



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Figure 2. Maximum-likelihood tree generated from the PB2 gene segment dataset of all seal viruses available, and their BLAST hits. Tip shapes and taxa names are coloured as follows: seal virus used as query—blue; BLAST hits that occur for a single segment only—grey; BLAST hits common to more than one internal gene segment are in progressively darker shades of pink as per key: 1 (=no repeats), 2, 3, 4, and 6. Trees for other internal gene segments can be found in [Supplementary Fig S5](#).



**Figure 3.** MCC summary trees for BEAST analysis of *A/grey\_seal/England/027661/2017* segment datasets: PB2, HA, and NA (A–C, respectively). Nodes connecting the seal virus tip with the closest-related strain(s) are highlighted in grey. Diamond (◆) shapes at nodes indicate posterior probability ranging from white (0) to black (1.0). Nodes of interest are labelled with node ages as inferred by BEAST. Tip shapes are triangles for the seal query sequence, and circles for BLAST hits. Tip shapes are coloured according to the host that the virus was isolated from: Seal—blue, Human—light blue, Anseriformes—orange, Galliformes—red. All trees were plotted in R (v3.6) using the ggtree package. MCC trees for all segments (apart from MP) can be found in [Supplementary Fig. S2](#); a maximum-likelihood tree for MP is shown in [Supplementary Fig. S3](#).

**Table 1.** TMRCA tables for (A) *A/Grey\_seal/England/027661/2017* and (B) *A/harbour\_seal/Massachusetts/1/2011*.

Segment	TMRCA	Lower 95 per cent HPD	Upper 95 per cent HPD	Posterior probability of node	Host	Country	Year	Subtype
<b>(A) <i>A/Grey_seal/England/027661/2017</i></b>								
HA	2012.45	2011.03	2013.74	1	Barnacle goose	Netherlands	2014	H3N6
NA	1999.13	1994.68	2002.72	0.6	Teal/common gull	Norway	2007/2009	H3N8/H6N8
MP	n/a	n/a	n/a	n/a	Duck	Netherlands	2010	H3N2
NP	2014.61	2013.94	2015.24	0.78	Mallard	Netherlands	2015	Unknown
NS	2009.65	2009.65	2010.71	0.5	Barnacle goose	Netherlands	2011	H6N8
PA	2013.59	2012.96	2014.16	0.43	Mallard	Netherlands	2014	H7N5
PB1	2014.11	2013.41	2014.83	1	Duck/mallard	Netherlands/France	2015	H5Nx
PB2	2011.91	2011.21	2012.85	0.59	Duck/chicken	France	2016/2015	Multiple
<b>(B) <i>A/harbor_seal/Massachusetts/1/2011</i></b>								
HA	2007.9	2004.27	2009.74	0.67	Blue-winged teal/mallard	New Brunswick/Ohio	2010/2011	H3N8/H3N2/H3N1
NA	2009.25	2008.92	2009.52	0.27	Northern pintail/mallard	Minnesota	2009	H3N8
MP	2009.97	2009.57	2010.49	0.52	American black duck	New Brunswick	2009	H4N6
NP	2009.3	2008.92	2009.59	1	American black duck	New Brunswick	2009	H4N6
NS	2009.57	2008.99	2009.7	0.1	American black duck	New Brunswick	2009	H4N6
PA	2009.35	2009.02	2009.61	0.93	American black duck	New Brunswick	2009	H4N6
PB1	2009.3	2008.85	2009.61	1	American black duck	New Brunswick	2009	H4N6
PB2	2008.95	2008.22	2009.46	1	American black duck	New Brunswick	2009	H4N6

Putative divergence times (TMRCA) and 95 per cent highest posterior density (HPD) of the seal sequence from the closest-related wild bird sequence for each segment, and the posterior probability of the node are shown, along with information about the closest-related wild bird sequence including the host, country, and year of isolation and subtype of virus. For the MP gene, information about the closest-related sequence according to the ML tree is shown.

**Table 2.** Amino acid substitutions table.

Segment	Amino acid substitutions in seal virus: A/Grey_seal/England/027661/2017	References	
HA	Y9C [signal peptide]		
	T64A [48]		
	I81V [65]	<a href="#">Anthony et al. (2012)</a>	
	S111G [95]	<a href="#">Liu et al. (2011)</a> (residue 110)	
	possible A154T (but might be same RCT → ACT (T), or GCT (A)) [138]		
	A/T176S [160]	<a href="#">Anthony et al. (2012)</a>	
	S235Y [219]		
	MP	None	
	NA	N/S41D	
		T383A	
NP	V104M		
NS	None		
PA	V91I		
	D/G101N		
PB1	N/S321I	<a href="#">Miotto et al. (2010)</a>	
	I517V	<a href="#">Tamuri et al. (2009)</a>	
	S678N	<a href="#">Gabriel et al. (2005)</a>	
PB2	T/A105K	<a href="#">Tamuri et al. (2009)</a> and <a href="#">Miotto et al. (2008)</a>	
	D161N		
	A/S395T		
	V667I		
	R/T676I		
	D701N	<a href="#">Liu et al. (2018)</a> and <a href="#">Steel et al. (2009)</a>	
	possible R753T (but might be same ASA → AGA (R) or ACA (T))		

Segment-wise list of substitutions found in the seal virus A/Grey\_seal/England/027661/2017 inferred from inspection of amino acid alignments of the seal virus sequence along with its 50 BLAST hits. Only residue changes that occur in the seal virus but not in the wild bird viruses are shown. Numbers in square brackets for HA indicate the reference H3 numbering of residues in A/Aichi/2/1968 ([Burke and Smith 2014](#)) found using the tool at FluDB website: <https://tinyurl.com/HANumbering>. Substitutions which have been previously described in a published study are shown in bold and their respective references are shown in the third column. For substitutions at previously documented residues, but resulting in different amino acids, only the residue number is shown in bold.

mammalian cells and showed that it has been associated with increased transmission in ferret experiments. Another mutation in the PB2 gene, at residue 105 in its NP-binding region ([Poole et al. 2004](#)) was also found in studies that used phylogenetic modelling ([Tamuri et al. 2009](#)) and mutual information statistics ([Miotto et al. 2008](#) 2010). Both the mutations found in the PB1 gene are also of interest. PB1-I517V was found by [Tamuri et al.](#) and S678N found in the seal PB1 gene has been associated with increased polymerase activity and virulence in mice ([Gabriel et al. 2005](#)).

Changes were also found in the HA gene (Table 2), but the implications are less clear. There appear to be no changes in the glycosylation patterns between the HA and NA of the Grey seal virus in comparison to related wild bird viruses (Supplementary Table S3A and B).

We compared all the substitutions with previously described mutations in seal influenza virus infections, and found that

apart from D701N, which was also found in the H3N8 seal virus infection in Massachusetts in 2011 ([Anthony et al. 2012](#)), there were no convergent amino acid changes. In the H3 HA gene, we found substitutions in residue 81 (reference H3 numbering—64), and residue 176 (reference H3 numbering—160), and these are also altered in the 2011 Massachusetts seal virus ([Anthony et al. 2012](#)) but to different amino acids. The latter mutation was not implicated in receptor binding for the seal viruses as was the case with H5N1 and some human H3N2 viruses, because the glycosylation site is absent in both seal and wild bird IAVs (Supplementary Table S3A). The HA of the 2011 Massachusetts seal virus had an F110S mutation, where the 110 residue has been previously found to be a critical component of the influenza viral fusion peptide, which may impact replication in mammalian cells ([Liu et al. 2011](#); [Anthony et al. 2012](#)). Our reported seal virus retains F at position 110, but whether the mutation in the adjoining residue at S111G (reference H3 numbering 95) has any effect on HA fusion properties is unknown. The presence of S at position 66 in the PB1 sequence, which enables production of PB1-F2 ([Conenello et al. 2007](#)) was found in the 2011 Massachusetts H3N8 Harbour seal virus but was not seen in this 2017 H3N8 Grey seal virus. Changes at positions 226 and 228 in HA (reference H3 numbering) which can change receptor-binding preferences between avian and mammalian hosts ([Connor et al. 1994](#); [Matrosovich et al. 2000](#)), were not found in either of the H3N8 seal viruses, but the H10 equivalent of H3-Q226L was identified in viruses from the 2014–15 H10N7 outbreak in European seals ([Dittrich et al. 2018](#)).

## 4. Discussion

### 4.1 IAV was not the cause of death in the Grey seal pup from which it was isolated

In the seal infection case reported here, the animal was referred to a rescue centre because it was stranded and the IAV was detected only incidentally. The vast majority of Grey seals admitted to rehabilitation centres in the UK are pups within the first year of life. Malnutrition is the single most common reason for pinnipeds to be taken into rehabilitation centres ([Barnett et al. 2000](#); [Van Bonn 2015](#)). Wounds are often recorded and hold clinical significance, as in this case, and have been considered predisposing factors for fatal non-specific septicaemia ([Baily 2014](#)). The cause of trauma and wounds may be anthropic (entanglement in fishing nets or gears) or biologic (conspecific aggressiveness or hierarchical to cannibalistic behaviours). Wounds caused by bites of other seals or predators were most often seen ([Barnett et al. 2000](#); [Van Bonn 2015](#)). Pulmonary and nasal parasites are also very well documented clinico-pathological conditions among rescued seal pups, and likely caused the hyperplastic rhinitis identified in this study seal. No histopathological findings consistent with IAV-derived damage were detected along the respiratory tract. The IHC demonstrated productive viral replication only in nasal mucosa, but not in lower respiratory regions, although the more sensitive RRT-PCR revealed presence of trace amounts of viral RNA within the lower respiratory tract, suggesting that the infection may have been cleared naturally or indicating a passive translocation of non-replicating virus nucleic acid fragments from upper respiratory regions. Efficient clearance in healthy Grey seals may explain why previous attempts at sequencing IAV identified during surveillance were unsuccessful ([Puryear et al. 2016](#)). This is consistent with previous studies which found that Grey seals were likely to remain sub-clinical during IAV infection while a proportion of the surveyed adults



and juveniles were seropositive (Bodewes et al. 2015; Puryear et al. 2016).

#### 4.2 Cross-species transmission dynamics of IAV

Puryear et al. discuss the possibility that Grey seals may be more prone to infection due to gain in population density since the marine mammal protection act in the USA in 1972, along with more socially gregarious and aggressive behaviour in comparison to Harbour seals, all of which contribute to high pathogen transmissibility. Why we see differences between Grey and Harbour seals in their resistance to diseases caused by viral agents is however unclear. Phylogenetic analyses have been unable to resolve the relationships between different seal species with sufficient support but Grey seals are either placed as a sister group to Caspian seals (*Pusa caspica*) and separate from Harbour seals, or in a basal position to both *Phoca* and *Pusa* genus (Fulton and Strobeck 2006; Berta, Churchill, and Boessenecker 2018). It might be informative to explore long-term evolutionary history of different seal species, including the effects of population bottlenecks and their impact on immune diversity, along with host physiology and immune response to understand differences in viral pathogenicity.

Our phylogeny and molecular clock analyses suggest different lineages of source viruses, and time of introduction of different segments of A/Grey seal/England/027661/2017 (with the caveat of being inferred for a single genome). It is not surprising to find that the closest-sampled and sequenced viruses for each segment are of different subtypes, hosts and times of isolation, given the relatively limited surveillance in wild birds, and the extensive reassortment (Wille et al. 2013; Lu, Lycett, and Leigh Brown 2014; Venkatesh et al. 2018), i.e. swapping of individual segments into different progeny viruses during propagation after mixed infection with two or more viruses. It would be plausible to propose a wild bird species as a likely donor, however. Studies on avian influenza viruses have previously shown that the transmission between birds is directional, e.g. usually in the direction from Anseriformes such as ducks into Galliformes (chicken) or Charadriiformes (gulls) (Venkatesh et al. 2018). In the case of this current infection, where we have found unambiguously closest-related sequences, they have tended to have been isolated from Anseriformes species. The above findings, along with the observation that the closest-related viruses were isolated largely from the Netherlands, it is likely that the source of the viral segments come from unsampled avian IAVs from an Anseriformes host within Northern Europe. The closest-related wild bird viruses to the A/harbour\_seal/Massachusetts/1/2011 virus were also isolated from Anseriformes species (American black duck) from New Brunswick/Ohio and Minnesota. In contrast with the Grey seal virus however, phylogenetic analysis reveals a restricted set of wild bird strains that are most closely related with Harbour seal virus, and mean TMRCAs from closest avian IAV segments that range within two years previous to isolation from harbour seal. In contrast, for the grey seal influenza virus, the range of TMRCAs from putative closest avian sequences for each gene segment is large, so it is difficult to infer anything about the timeframe(s) of avian-to-seal transmission event(s), or estimate how long the virus has been circulating in seals. The level of surveillance and genome sequencing of IAV in seals is very low, and usually only tends to be done during high mortality outbreaks. Surveillance of healthy seals, especially those that tend to remain sub-clinical, might shed more light on transmission dynamics between seals and other species, and possibly predict outbreaks and mass die-offs in susceptible species. Previous studies in North America have

indicated that Grey seals are possibly a reservoir for IAV and other viruses (Duignan et al. 1995, 1997; Puryear et al. 2016), and our results are consistent with this hypothesis.

#### 4.3 Molecular signatures of mammalian adaptation in avian-derived seal influenza viruses

In our dataset of closest-related sequences to the seal virus, we find that while there are several substitutions found in the seal virus which do not occur in the bird viruses; we generally do not find substitutions in any of the bird IAVs that do not occur in other bird viruses too. This likely indicates adaptation to the seal environment, a hypothesis supported by the occurrence of the D701N mutation, a known rare marker of mammalian adaptation (Steel et al. 2009; Liu et al. 2018). D701N is common in canine and horse H3N8 IAV (see Supplementary Table S4), but does not occur in birds, and was found associated with highly-pathogenic H5N1 viruses which infected humans (Gabriel et al. 2005; Li et al. 2005; de Jong et al. 2006). D701N mutation was also found in the A/harbour\_seal/Massachusetts/1/2011, but not in the H10N7 outbreak, despite sustained transmission in seals for several months, nor in any other previously sequenced seal PB2 genes. H3N8 IAV are noted for the ability cross-species barriers, so it would be relevant to consider if the subtype of the virus has any influence on the kind of adaptive mutations that occur in the polymerase genes, and if so what sort of mechanisms this might involve. We also note that many of the putative adaptive substitutions occur in the polymerase complex genes, which are increasingly being recognised for their vital role in mediating viral host range (in addition to receptor compatibility with glycoproteins).

The 2011 North American H3N8 and 2014 European H10N7 viruses which caused outbreaks in Harbour seals were found to have acquired mutations to enable recognition of sialyloligosaccharide receptors found more abundantly in mammalian tissues (SA $\alpha$ 2,6Gal) but which retained the ability to interact with avian receptors (SA $\alpha$ 2,3Gal). Another study (Karlsson et al. 2014) also found that the 2011 virus displayed increased affinity for mammalian-type receptors in *in vitro* glycan binding assays, was able to replicate in human lung tissues, and transmit via respiratory droplets between ferrets. A later detailed structural and *in vitro* functional analysis of the 2011 H3N8 seal HA indicated a true avian receptor binding preference (Yang et al. 2015), as did a mutational analysis of the H10N7 viruses (Dittrich et al. 2018). It therefore appears that the viruses are able to replicate in and transmit between mammals (seals and ferrets) while retaining structurally 'true avian-type' receptor interaction. It is likely that virally and host receptor factors beyond solely the SA $\alpha$ 2,3Gal/SA $\alpha$ 2,6Gal linkages and HA binding determine host restriction of IAVs, and further investigation into the relationship between receptor specificity and transmission across species will shed light on these factors (Gulati et al. 2013; Walther et al. 2013; Air 2014; de Graaf and Fouchier 2014; Jia et al. 2014; Byrd-Leotis, Cummings, and Steinhauer 2017).

Although there are some common HA residues that are changed in both the 2017 H3N8 seal virus reported here and other avian-derived seal IAVs, we found limited convergence in the substitutions and residues involved between the different seal viruses. Of the twenty-two substitutions found in A/Grey seal/England/027661/2017, the number is highest in HA and PB2 segments (seven each), which is consistent with the critical role of these proteins in host restriction/adaptation. Anthony et al. (2012) report a total of thirty-seven amino acid substitutions in the harbour seal H3N8 virus in comparison with related avian

IAVs, with the highest number occurring in HA and NA (11 and 7, respectively). We therefore see different pathways to mammalian adaptation on spill-over from avian sources—likely attributable to different viral genetic backgrounds. In addition, to the best of our knowledge, ours is the first Grey seal virus sequence that is being made publicly available (via GISAID). It is therefore uncertain whether the type of mutations occurring in a putative reservoir host vis-à-vis avian-like sequence might be different from those occurring in related hosts with pathogenic outcomes.

H3N8 viruses currently circulate in horses but not humans or pigs. However, H3 viruses with other NA combinations have been found in several species including humans, pigs, horses, dogs, cats, seals, poultry, and wild aquatic birds. Therefore, H3 viruses have been noted for a particular ability to cross-species barrier and cause productive infections. One study that examined the ability of H3N8 viruses from canine, equine, avian, and seal origin to productively infect pigs, demonstrated that avian and seal IAVs replicated substantially and caused detectable lesions in inoculated pigs without prior adaptation (Solórzano et al. 2015). It is possible that the ready occurrence of PB2 701N mutation in H3N8 viruses contributes to this ability. We do not have any biological evidence for pre-twentieth century human IAVs, but historical analysis suggests a long association of humans with influenza (Hirsch 1883; Taubenberger and Morens 2010), and has uncovered temporal–geographic associations between equine and human influenza-like disease activity documented in Europe in the sixteenth to eighteenth centuries (Morens and Taubenberger 2010). Such analysis has also implicated an H3N8 virus in the 1889 pandemic in humans (Morens and Taubenberger 2010), which makes mammalian-adapted H3N8 viruses of particular interest as IAV pandemic risk candidates.

In this article, we have presented analyses of a case of seal infection with IAV in coastal England and compared it with previously reported seal IAV infections. This infection provides a small but unique window to understand the ecology of avian-origin IAVs that may be circulating and maintained in mammals. Given the mammalian adaptation activity in IAV upon transmission to seals, such infections may be of interest to pandemic surveillance and risk and help us better understand the determinants of mammalian adaptation in influenza viruses and its complex drivers.

## Supplementary data

Supplementary data are available at Virus Evolution online.

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