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Research paper

Urbanization and the dynamics of RNA viruses in Mallards (*Anas platyrhynchos*)

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ABSTRACT

Urbanization is intensifying worldwide, and affects the epidemiology of infectious diseases. However, the effect of urbanization on natural host-pathogen systems remains poorly understood. Urban ducks occupy an interesting niche in that they directly interact with both humans and wild migratory birds, and either directly or indirectly with food production birds. Here we have collected samples from Mallards (*Anas platyrhynchos*) residing in a pond in central Uppsala, Sweden, from January 2013 to January 2014. This artificial pond is kept ice-free during the winter months, and is a popular location where the ducks are fed, resulting in a resident population of ducks year-round. Nine hundred and seventy seven (977) fecal samples were screened for RNA viruses including: influenza A virus (IAV), avian paramyxovirus 1, avian coronavirus (CoV), and avian astrovirus (AstroV). This intra-annual dataset illustrates that these RNA viruses exhibit similar annual patterns to IAV, suggesting similar ecological factors are at play. Furthermore, in comparison to wild ducks, autumnal prevalence of IAV and CoV are lower in this urban population. We also demonstrate that AstroV might be a larger burden to urban ducks than IAV, and should be better assessed to demonstrate the degree to which wild birds contribute to the epidemiology of these viruses. The presence of economically relevant viruses in urban Mallards highlights the importance of elucidating the ecology of wildlife pathogens in urban environments, which will become increasingly important for managing disease risks to wildlife, food production animals, and humans.

1. Introduction

Urbanization is intensifying worldwide; most humans live in urbanized areas, and the urban human population is expected to continue to grow (United Nations Population Fund, 2007). Within the global growth of cities, urbanization increasingly shapes the emergence and trajectory of infectious disease, both human disease and disease and parasitism in wild animals (Alirol et al., 2011; Neiderud, 2015). In association with urbanization, factors affecting pathogen (and parasite) transmission in wild animals include an increase in aggregation and resource availability resulting in increased contact rates, decrease in biodiversity, modulation in host immunity and stress levels (Becker and Hall, 2014; Becker et al., 2015; Bradley and Altizer, 2006; Delgado and French, 2012; Patz et al., 2004; Penczykowski et al., 2014). Furthermore, in cities, increased contact among humans, domestic animals and wild animals may facilitate cross species spillover of (vertebrate) pathogens, with consequences for wildlife conservation, agriculture, and human health (Becker et al., 2015; Bradley and Altizer, 2006; Delgado and French, 2012; Patz et al., 2004).

Influenza A virus (IAV) is a multi-host virus, wherein spillover between birds, humans and agricultural animals does occur, and dabbling ducks, such as those found in city parks, constitute the main reservoir host for these viruses (Olsen et al., 2006; Webster et al., 1992). Indeed, highly urbanized areas may contain canals and large city parks with ponds housing a wide variety of wild and semi-domestic birds. RNA viruses such as IAV have a low pathogenicity phenotype in their natural hosts (Olsen et al., 2006), but have large negative socioeconomic consequences when they spillover into food production animals and humans (Alexander and Brown, 2009; FAO, 2005, 2012). For example, the most recent remerging highly pathogenic IAV H5N8, which was transported globally by waterfowl, resulted in the culling of hundreds of thousands of chickens and turkeys, and is a risk to human health given the reassortment potential (European Food Safety Authority, 2014; Lee et al., 2014; Pasick et al., 2015; Verhagen et al., 2015; Wu et al., 2014). Dabbling ducks are a host for a number of RNA viruses, including avian

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coronavirus [CoV], avian paramyxovirus type 1 [APMV-1], and emerging evidence suggests they may also be hosts for an array of avian astroviruses [AstroV] (e.g. Chu et al., 2012; Ramey et al., 2013; Tolf et al., 2013b; Wille et al., 2015; Wille et al., 2016b). These viruses do not cause signs of disease in their wildlife hosts, but have closely related forms causing morbidity and mortality in poultry, such as infectious bronchitis (CoV) (e.g. Domanska-Blicharz et al., 2014; Jackwood et al., 2012; Zhuang et al., 2015), Newcastle disease (APMV-1) (e.g. Alexander, 2011; Jindal et al., 2009; Ramey et al., 2013; Snoeck et al., 2013; Tolf et al., 2013b), duck hepatitis (AstroV) or avian nephritis (AstroV) (e.g. Chu et al., 2012; Pantin-Jackwood et al., 2011). These viruses have been assessed, to various degrees, in wild migrating waterfowl. In Sweden, and globally, the ecology of IAV is well described in wild waterfowl, where up to 30% of Mallards (*Anas platyrhynchos*) are infected during the autumn migration (Latorre-Margalef et al., 2014; Olsen et al., 2006). Recent studies have been instrumental in starting to describe dynamics and ecology of AMPV-1 and CoV in wild birds; 9–12% of migrating Mallards have CoV infections, compared to a lower prevalence (2%) of AMPV-1 towards the end of the migratory season in Sweden (Tolf et al., 2013b; Wille et al., 2015). Most APMV-1 is detected during IAV studies where agglutinating agents are detected after culture that are not IAV (e.g. Jindal et al., 2009; Ramey et al., 2013), so few true prevalence estimates exist. Beyond these viruses, we have a limited understanding of the virodiversity in waterfowl; astroviruses for example have only recently been assessed in wild birds, and the results of a single study suggest that waterfowl may be important in the epidemiology of these viruses (Chu et al., 2012). Given that waterfowl are hosts for both multi-host viruses and viruses that cause morbidity and mortality in food production birds, combined with the increased contact between waterfowl and humans in urban areas, dynamics of these viruses in urban bird populations should be explored.

In this study, we followed the dynamics of RNA viruses at a pond utilized year round by Mallards, located in the centre of Sweden's fourth largest city. This pond is on the same migratory route as wild Mallards assessed for these viruses in southern Sweden, allowing a comparison between urban and wild ducks on a limited spatial scale (Latorre-Margalef et al., 2014; Tolf et al., 2013a; Tolf et al., 2013b; Wille et al., 2015; Wille et al., 2016b). Thus, this intra-annual dataset allows us to add to the natural history of IAV, CoV, AMPV-1, and the rarely assessed AstroV. Furthermore, we aim to elucidate if less frequently studied RNA viruses follow intra-annual cycles similar to that of the intensively studied IAV. In context of IAV, and to a lesser degree CoV and APMV-1, an assessment of virus prevalence and diversity in an urban population will further allow us to assess if dynamics in wild birds are reflected in an urban setting.

2. Methods

2.1. Sample collection

An urban population of Mallards residing in the artificial pond "Svandammen" in the centre of the city of Uppsala, Sweden (59°51′16″N, 17°38′25″E) were sampled. The pond is kept ice-free during the winter months, and is a popular location where the ducks are fed, resulting in a resident population of ducks year-round. This pond has a largely constant population size between 300 and 600 individuals through the autumn and winter, with fewer birds occupying the pond during breeding in the summer months (Fig. A.1). The low population count in May is likely the result of unfavorable conditions on the day of the count and sampling. Slightly higher population counts in the winter, when most of the city ponds are frozen, likely represent the congregation of birds from ponds across Uppsala to utilize this ice-free habitat (Fig. A.1).

Two sampling strategies were employed: following capture, freshly deposited feces were collected from a single-use cardboard box, or, due to difficulties in capturing birds, freshly deposited feces were collected from the ground around the perimeter of the pond. Samples were collected with a sterile tipped applicator, and were placed in virus transport media (VTM) and stored at −70 °C within 2–6 h of collection.

Ethical approval for trapping and sampling was obtained from the Uppsala animal ethical committee (Reference Number C228/12), a permit was obtained from the City of Uppsala to capture, and a permit from Swedish Museum of Natural History to ring birds.

2.2. Sample screening

Viral RNA was extracted from pooled VTM samples, containing 4 samples per pool, with the Magnatrix 8000 extraction robot (Magnetic Biosolutions, Sweden) and Vet Viral NA kit (NorDiag ASA, Oslo, Norway). The RNA extraction was performed by the Molecular Diagnostics Department at the Swedish National Veterinary Institute. Positive pools were re-extractioned individually using the Maxwell 16 Instrument and Viral Total Nucleic Acid Purification Kit (Promega, Madison, USA). Following extraction, samples were assayed by real time reverse transcriptase PCR (rRT-PCR) for IAV, CoV, and AMPV-1 using previously published methods. Briefly, IAV was screened using a rRT-PCR assay targeting a short region of the matrix gene (Spackman et al., 2002) and a pan-coronavirus rRT-PCR assay targeting the RNA-dependant RNA polymerase (RdRp) gene (Muradrasoli et al., 2009) using the iScript One Step RT-PCR Kit (BioRad, Hercules, USA). A rRT-PCR targeting the matrix (M) gene (Tolf et al., 2013b; Wise et al., 2004) with the One Step RT-PCR Kit (Qiagen, Hilden, Germany) was employed to screen for APMV-1. A cycle threshold (Ct) cutoff of 40 was used for all screens. To screen for AstroV, cDNA was synthesized using Superscript III (Invitrogen) and random hexamers (Invitrogen) followed by a nested PCR targeting the RdRp (Chu et al., 2012; Chu et al., 2008) using Taq polymerase (Qiagen).

2.3. Virus characterization

Samples positive for IAV were propagated in 10–11 day old embryonated chicken eggs. Eggs were inoculated via the allantoic route, and allantoic fluid was harvested two days following inoculation. The fluid was assayed for the presence of IAV using a haemagglutination assay. RNA was extracted from positive samples as previously described. Egg isolation and extractions from allantoic fluid were performed by the Molecular Diagnostics Department at the Swedish National Veterinary Institute. Full length HA, NA, and M sequences were generated as described in Wille et al. (2013), and two samples were additionally deep sequenced in-house at the Swedish National Veterinary Institute (Virus 540/H10N3 and 816/H1N1).

A fragment of the CoV RdRp was sequenced as described in Wille et al. (2016b). The RdRp fragment generated during screening of AstroV was used and subsequently cloned with pGEM-T easy vector system (Promega). All PCR products were purified by the Wizard Clean-Up System (Promega) and all sequencing was completed at Macrogen (The Netherlands). In the case of astroviruses, 3–5 clones of each sample were sequenced.

Resulting sequences were aligned using the MAFFT algorithm (Katoh et al., 2009) within Geneious 7 (Biomatters, New Zealand). Phylogenetic models were determined in MEGA 6 (Tamura et al., 2011), and Maximum Likelihood Trees were built using PhyML (Guindon and Gascuel, 2003) implemented in SeaView (Gouy et al., 2010) and bootstrapped 10,000 times. Reference sequences for phylogenetic analysis comprised of the top BLAST hits for each sequence generated in this study, as well as similar sequences from Sweden. Outgroup sequences were added to root all trees. All sequences generated in this study have been deposited in GenBank under the accession numbers KY320400-39.

2.4. Statistics

Seasonal prevalence for each virus was estimated using Generalized Additive Models (GAMs) with binomial errors including a spline function of month using the mgcv package in R (R Development Core Team, 2008). The best order polynomial was evaluated through Akaike information criterion (AIC) and given similar AIC values the least complex model was selected (Table A.1).

Prevalence estimates of IAV, CoV and APMV-1 from this study were compared to those from Wille et al. (2015), wherein prevalence for these viruses was estimated in wild migratory Mallards across the autumn season. We compared data from Sept-Dec, which represents large sample sizes in both studies. Prevalence data were compared with Fisher Exact Tests for the four RNA viruses for each month. p-values of <0.05 were taken to indicate a significant difference in the compared proportions.

3. Results

3.1. Study population and sampling effort

Over the course of 13 months, 977 samples were collected from Mallards. Most of the samples collected were freshly deposited feces from the ground ($n = 912$), though 65 samples were fecal samples collected from captured birds. During the autumn months 100 samples were collected each month, with smaller sample sizes in the spring and winter (Fig. A.1).

3.2. Prevalence and effect of urbanization in IAV, CoV, APMV-1

Prevalence of IAV, APMV-1 and CoV were low, with an overall prevalence of 1%, 1%, and 0.3% respectively, across the intra-annual sampling regime. As expected, prevalence for IAV peaked during the autumn months, with only a single positive fecal sampled collected from the ground outside this period, in April. Seasonal prevalence of other RNA viruses mirrored patterns of IAV, with a prevalence peak in the autumn through to the early winter, as well as a detection in April; CoV in particular had a very similar prevalence curve to IAV in both the temporal trend and amplitude. Prevalence of APMV-1 was low, even in the autumn, with a single detection in August, November and December. Interestingly, both IAV and CoV were detected in April, and this did not represent a co-infected sample (Fig. 1).

In comparing prevalence [Sept-Dec] between our urban dataset and a wild bird dataset from southern Sweden using the same qPCR methods (Wille et al., 2015), autumnal prevalence for IAV ($p < 0.0010$) and CoV ($p < 0.0010$) is significantly different, where prevalence for both these viruses is lower in urban Mallards (Fig. 2). A more detailed comparison suggests that this effect is strongest in Oct/Nov ($p < 0.0010$) for IAV and Sept/Oct ($p = 0.0036$, $p = 0.0049$) for CoV. Total autumnal prevalence, and monthly comparisons are not significantly different between these datasets for APMV-1, but this is driven by sample size constraints (Fig. 2). Timing of prevalence peaks do vary across years, that is the prevalence peak may occur in a different month across years. However, the difference in prevalence between urban and wild ducks does not appear to be driven by a temporal mismatch in prevalence peaks. Rather, the overall amplitude of the prevalence curves across the entire autumn for wild ducks and urban ducks are different, where the urban ducks consistently had lower prevalence for IAV and CoV (Fig. 2).

3.3. Diversity and characterization of IAV and CoV

Overall for both IAV and CoV there was detected diversity, irrespective of lower prevalence compared to the wild migratory bird system. Despite few IAV detections, five subtype combinations were detected: H1N1, H3N8, H6N4, H10N3 and H10N4. Furthermore H3 and H10, representing Group 2 HA, were detected earlier in the season (September), followed by H6 and H1, Group 1 HA viruses, in October and December, respectively (Table A.2). Genetically the HA segment of H6 and H10 were similar to viruses previously detected in Europe, including Sweden (Fig. A.4–A.5). Specifically the HA segment of H6 falls into a mixed clade containing viruses from Europe, Asia and North America (Fig. A.4). The HA of H10 is similar to sequences from Sweden, the Netherlands, as well as Egypt, and the Republic of Georgia (Fig. A.5). The HA segment of both H3 and H1 fall into clades dominated by Asian viruses (Fig. 3, Fig. A.2–A.3). The H1N1 virus is further interesting as the NA

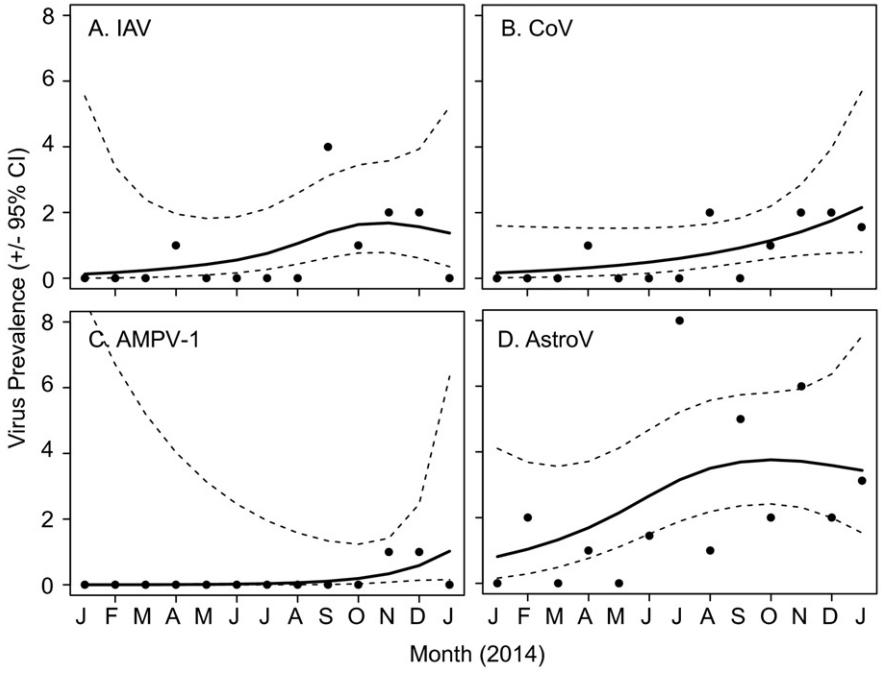

Fig. 1. Seasonal prevalence of RNA viruses (a) IAV, (b) CoV, (c) AMPV-1, and (d) AstroV. The predicted prevalence curve of each virus (solid line) and 95% confidence intervals (dotted lines) is shown, and model selection parameters are presented in Table A.1. Actual prevalence estimates for each month are plotted as points.

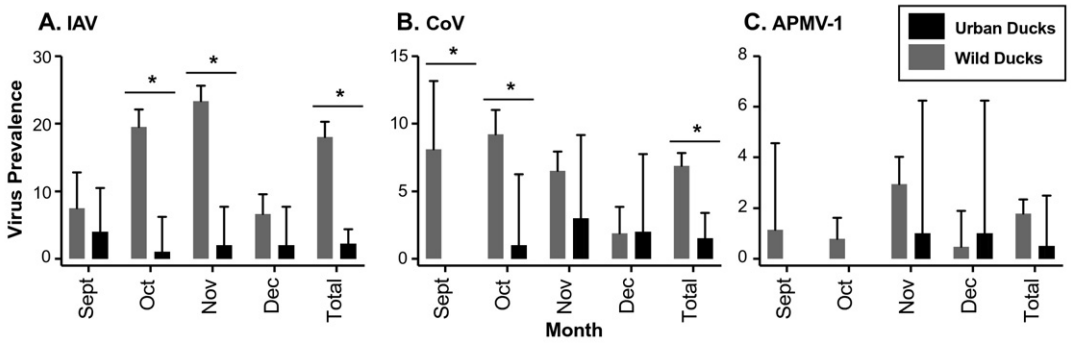

Fig. 2. Comparisons of autumnal prevalence for (a) IAV, (b) CoV and (c) APMV-1 between urban ducks (this study) and wild migratory ducks (Wille et al., 2015). Mean and 95% confidence intervals shown for prevalence, and asterisks indicate a significant difference. "Total" here is the combined number of viruses detected/total samples screened for the months of September, October, November and December.

segment and the M segment are also most similar to Asian viruses (Fig. 3); as compared to the N3, N4 and N8 sequences and the M segment of the other viruses (Table A.3, Fig. A.6). All N4 sequences were identical, despite being detected in two different months and with different HA types (H10N4 in September and H6N4 in October) (Table A.3). Finally, the M segment of all viruses except H1N1 were highly similar (Fig. A.6).

Similarly, a diversity of CoV RdRp fragments was present in this population (Fig. 4). All viruses were identified as gammacoronaviruses, and fell into the clade dominated by wild bird viruses and those recovered from domestic ducks. Some sequences generated here were very similar to those from Mallards migrating through southern Sweden in 2011 (Virus 485, 937). But, virus 271 and 665 were most similar to sequences from waterfowl coronaviruses isolated in Hong Kong. Most interestingly, virus 271 and 665 were identical, despite being isolated 6 months apart (14 April and 13 October 2014, respectively), suggesting RdRp sequences falling into this clade were present in Sweden despite not being previously detected (Fig. 4). We were unable to sequence APMV-1 given we were unable to culture these viruses and the original material had high Ct values.

3.4. Prevalence and diversity of AstroV

Against expectation, the highest RNA virus prevalence in this study was that of AstroV (Fig. 1D). Furthermore, we detected AstroV in 9/13 months, making it more pervasive than IAV in this population. However, prevalence did follow the general seasonal trend where most detections occurred in the autumn migration period (Fig. 1D). Additionally, 5/6 co-infected samples in the entire dataset were co-infected with AstroV (AstroV:Flu = 3, AstroV:CoV = 2, CoV:APMV-1 = 1).

As with the other viruses, there was a diversity of AstroV in the population (Fig. 5). Indeed, we identified viruses in all three branches of the avian AstroV tree. The virus detected in Group 2 - that is viruses similar to avian nephritis virus - (Virus 313) was the outgroup to this clade, suggesting undiscovered diversity, potentially in the wild bird reservoir. We similarly found an outgroup virus to Group 3 viruses (Virus 509), which are wild bird astroviruses detected thus far only in waterfowl. Two other viruses also fell into Group 3. Most of the viruses sequenced were Group 1 viruses, both Group 1.2 and 1.3, and most viruses were similar to duck hepatitis viruses (EU669004 duck hepatitis virus 3 and EU669003 duck hepatitis virus 2). Viruses were also similar to turkey astrovirus 2 (Virus 137; e.g. DQ066581) and chicken astrovirus (Virus 553; e.g. EU668998). Our preliminary findings suggest that Group 1.2 was more common early in the year (February, April, August) and Group 1.3 was more common towards the end of the study (September, November, December, January) (Fig. 5b), however a larger dataset is needed to confirm this putative trend.

4. Discussion

4.1. Effect of urbanization on RNA virus dynamics

Urbanization is intensifying world-wide, directly affecting interactions between humans and wild animals, in particular wild animals utilizing urban environments. In this study we aimed to characterize the dynamics of four avian RNA viruses in wild birds utilizing an urban environment. These viruses, while causing no apparent clinical signs in wild waterfowl, are closely related to or have pathogenic variants, which may cause significant morbidity and mortality in poultry. In wild birds, especially waterfowl, IAV has been intensively assessed, and we found that in an urban environment, annual dynamics of IAV was similar to the global consensus. That is, very low prevalence in the spring and summer, with a higher prevalence in autumn and early winter when birds are migrating (Olsen et al., 2006). In this study prevalence is lower in urban ducks than wild migrating ducks, here significantly lower than wild ducks utilizing a stop-over site in southern Sweden (Latorre-Margalef et al., 2014; Wille et al., 2015). There is some evidence that prevalence of IAV might be lower in urban and sentinel populations, but this still needs more expansive assessment. Verhagen et al. (2012) demonstrated that IAV prevalence is inversely correlated with urbanization, and urban Mallard prevalence is only 1.9%, which corroborates our findings. However, the bird species composition and temporal sampling between urban and rural areas were mismatched (Verhagen et al., 2012). In another study, in eastern Canada, prevalence of largely non-migratory urban ducks in the city of St. John's, Newfoundland, was 7.2%, with higher prevalence only reported when samples sizes were small (Huang et al., 2013a). One hypothesis for low viral prevalence in urban areas is host population structure and migratory propensity. Concentrated resources presented in urban environments influence host migration and among/between species contact rates (Altizer et al., 2011; Bradley and Altizer, 2006). Specifically, a larger proportion of urban ducks are non-migratory, although local movements do occur, particularly during breeding. Due to a more sedentary lifestyle, following the initial input of susceptible ducklings after breeding, there is limited immigration, representing input of susceptible individuals across the autumn. In contrast, at a migratory stopover location such as Ottenby, there is continual input of new individuals across the season representing both susceptible and infected birds (Latorre-Margalef et al., 2014). The continual immigration creates a constant pool of susceptible birds and input of diverse HA subtypes. Emigration allows for the removal of recovered birds from the system, allowing for higher viral prevalence across the autumn migration (Altizer et al., 2011; Avril et al., 2016). Lack of migration is also a feature of sentinel ducks, and prevalence and viral diversity was low in sentinel ducks being assessed on Lake Constance (Globig et al., 2009; Globig et al., 2012) and adult sentinel ducks in Sweden (Tolf et al., 2013a). An interesting parallel is IAV dynamics in Africa where IAV seasonality in

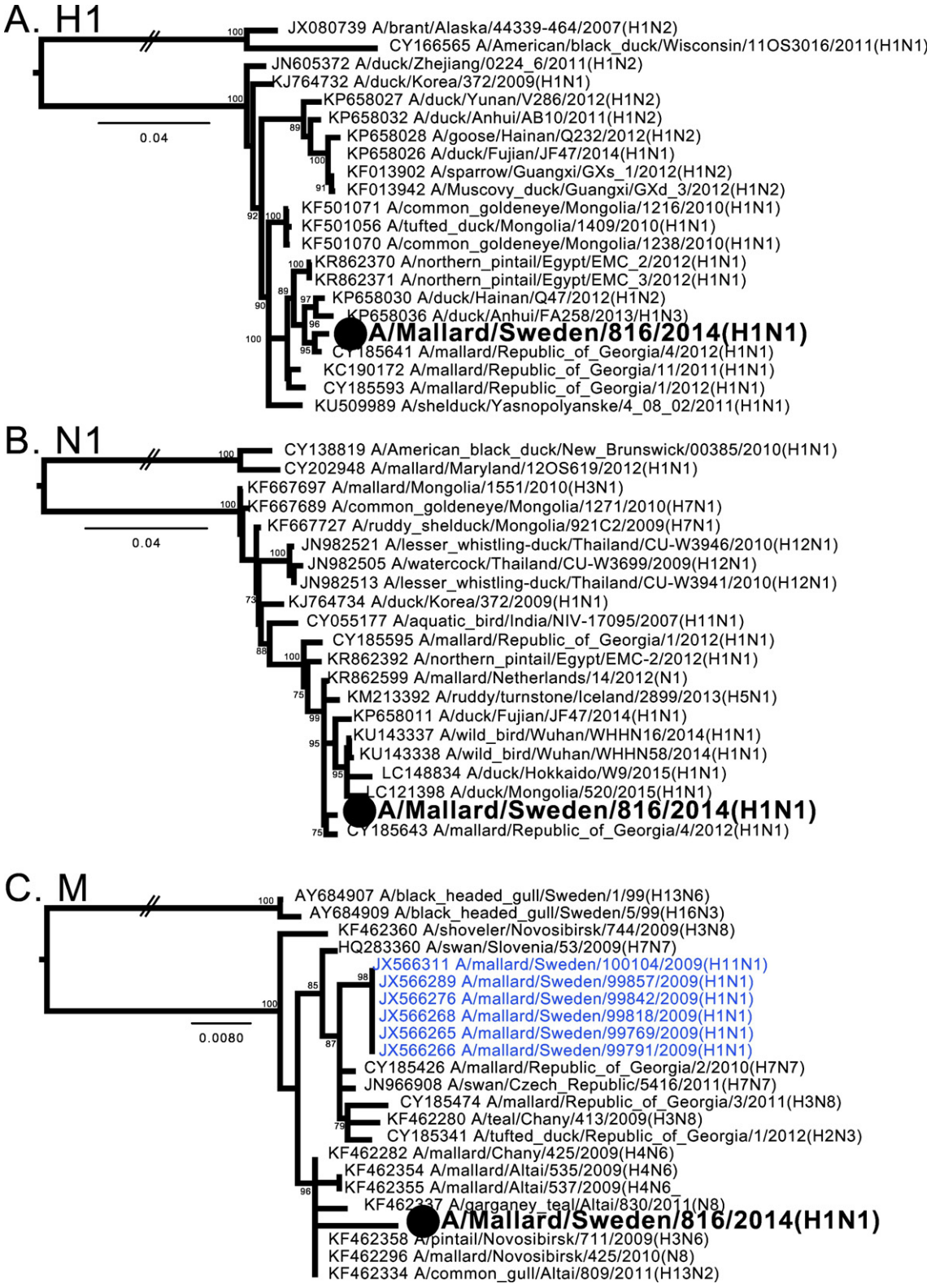

Fig. 3. Asian-like H1N1 influenza A virus isolated from ducks in December 2014 at Svandammen. Full length (a) HA, (b) NA, and (c) M segment, top 20 BLAST hits, and two outgroup sequences are included in each tree. Sequences generated in this study are indicated with a filled circle. Viruses isolated previously in Sweden are coloured in blue. Scale bar represents number of substitutions per site. More expansive H1 and M tress are presented in Fig. A.2 and Fig. A.6, respectively.

muted, and prevalence is very low. The putative driver is different life history strategies of waterfowl, wherein the classical patterns of waterfowl aggregation and migration in temperate regions are less pronounced, with only the subset of Palearctic breeding waterfowl exhibiting long distance migration; Afro-tropical waterfowl are resident or partial migrants likely due to more abundant resources. Furthermore, an increase in IAV prevalence was correlated to the influx of Palearctic migrants (Gaidet et al., 2012; Gaidet et al., 2007). In terms of population

Fig. 4. RNA-dependent RNA polymerase (RdRp) of avian coronaviruses from urban Mallards are diverse. Viruses isolated previously in Sweden are coloured in blue. Sequences generated in this study are indicated with a filled circle. Infectious bronchitis virus and Turkey Coronavirus, both gammacoronaviruses, are set as the outgroup. Scale bar represents number of substitutions per site.

structure, urban ponds in Uppsala are utilized by a single dabbling duck species - Mallards; other dabbling duck and waterfowl species are absent limiting the breadth and size of the host reservoir. Finally, urban Mallards have access to more resources than their wild conspecifics due to supplemental feeding, which in turn may allow these individuals to mount a more efficient antiviral response (Chandra, 1999; Hall et al., 2009), at both the innate (antiviral genes more highly unregulated) (Barber et al., 2008; Vanderven et al., 2012) and acquired level (length of antibody life) (Magor, 2011). Unfortunately, there are few studies assessing the antiviral response to IAV, and those that do exist are largely focussed on the response to highly pathogenic IAV (Barber et al., 2008; Huang et al., 2013b; Vanderven et al., 2012). Interestingly, despite the potential for an improved immune response, some studies suggest that an increase of resource may increase transmission potential and pathogen transmission (Becker and Hall, 2014; Penczykowski et al., 2014). Despite empirical studies suggesting lower prevalence of IAV in urban systems, however limited, theoretical studies imply that pathogen prevalence should be higher in these conditions (e.g. Hall et al., 2014). These theoretical studies have been verified by empirical work. For example, in Monarch butterflies (*Danaus plexippus*) that have lost migratory behaviour there is an increase in infection risk of a protozoan parasite (Satterfield et al., 2015). The reason for this conflict is unknown, however, one hypothesis is that these studies utilize a chronic disease model, whereas influenza is an acute infectious disease, and dynamics are driven largely by the herd immunity of the population (Latorre-Margalef et al., 2014; van Dijk et al., 2014; Wille et al., 2016a). There are a number of factors which may be important drivers in dynamics of diseases in urban environments, including the relationship between provisioning, stress, pollution and immune response which affect susceptibility and ability to fight infection, however these are challenging to disentangle (Becker et al., 2015; Bradley and Altizer, 2006; Delgado and French, 2012; Patz et al., 2004), and these factors in relation to RNA virus dynamics need to be assessed.

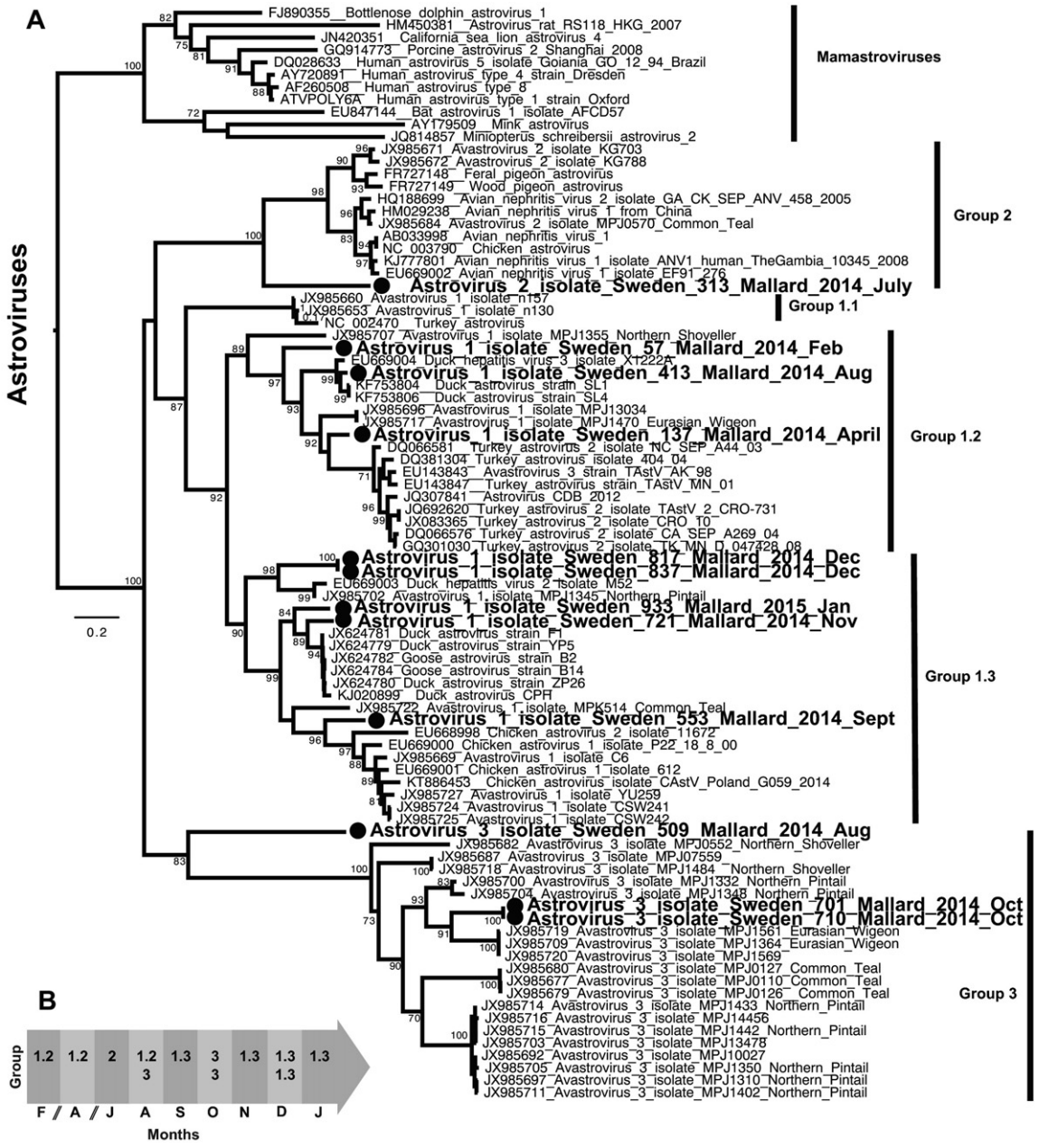

Fig. 5. RNA-dependent RNA polymerase avian astrovirus diversity in urban Mallards. (a) Phylogenetic tree of astroviruses. Sequences generated in this study are indicated with a filled circle. Mamastroviruses are set as the outgroup. Scale bar represents number of substitutions per site. (b) A depiction of the astrovirus group, by month, detected in sequenced viruses from urban Mallards.

4.2. Conserved trends across RNA viruses

While IAV has been intensively assessed, we are only starting to explore dynamics of other avian RNA viruses. Indeed, this is the first intra-annual dataset exploring dynamics of CoV, APMV-1 and AstroV and, furthermore, the first comparison between wild and urban settings for CoV and APMV-1. It is also the second study assessing AstroV in wild birds (Chu et al., 2012). Given the economic implications of these viruses, our limited understanding of the dynamics and ecology of these viruses in wild birds is disquieting. Perhaps unsurprising, overall annual trends in prevalence were similar for all viruses, and shared ecological drivers, such as those identified for IAV (van Dijk et al., 2014), are the most parsimonious explanation for the shared patterns in long term dynamics of these viruses. That is, increased prevalence due to input of immunologically naïve birds into the system after breeding and aggregating of birds for autumn migration, and decreased prevalence in the winter following an increase in herd immunity (Latorre-Margalef et al., 2014; Olsen et al., 2006; van Dijk et al., 2014). This dataset provides further evidence of the importance of waterfowl, urban or wild, in the epidemiology of CoV and APMV-1. It is only within the last 10 years that CoV have been assessed in wild birds and these viruses have largely been assessed using single time point studies, across a range of species, and using an array of different screening methods (e.g. Chu et al., 2011; Muradrasoli et al., 2010; Wille et al., 2016b). Given the long history of APMV-1 research (Alexander et al., 2012), and "accidental" isolation of this virus in IAV studies (e.g. Jindal et al., 2009; Ramey et al., 2013), it is known that these viruses are present in waterfowl, however accurate prevalence estimates are still rare (Tolf et al., 2013b; Wille et al., 2015). Not all economically relevant avian RNA viruses have been assessed in wild birds, and astroviruses are such an example. Most strikingly, in this population, AstroV might be more pervasive than IAV, which has long been thought to be one of the most important RNA viruses in wild waterfowl.

Not only was prevalence of AstroV higher than IAV, viruses were detected over a longer temporal interval. These viruses are particularly interesting due to the importance in poultry including chickens, turkeys and ducks, but the overall lack of assessment in wild birds leads to limited understanding of the epidemiology and ecology beyond food production birds. Furthermore, this study corroborates Chu et al. (2012) in that wild birds appear to contribute to the epidemiology of chicken or turkey "adapted" astrovirus strains.

Not only was the overall prevalence trend conserved across all viruses, the prevalence difference between wild and urban birds was also conserved for CoV. This relationship between CoV and IAV, illustrated here by similar trends in urbanization could be due to a mutualistic relationship, that is prevalence of CoV in waterfowl has been shown to be higher given infection with IAV in wild migrating Mallards (Wille et al., 2015). Prevalence for APMV-1 was not significantly different between this urban population and a wild migratory population, however this could be driven by sample size constraints – that is for a disease with a prevalence of <2% a much larger sample size is required to adequately assess prevalence with confidence (Hoye et al., 2010). Furthermore, given the scarcity of prevalence studies in wild birds and diversity of methods used, it is not certain if this trend in APMV-1 is due to methodological constraints or whether there are different drivers of APMV-1 ecology. Indeed, there might be an inverse relationship between APMV-1 and IAV prevalence, where APMV-1 prevalence increases when IAV prevalence decreases in wild Mallards (Tolf et al., 2013b; Wille et al., 2015). Despite the factors associated with urbanization, this overall seasonal trend for these viruses, likely driven by shared ecological factors, remains clear.

4.3. Perspective

This study highlights our limited understanding of RNA virus dynamics in birds in general, and more specifically, viruses in Mallards. Mallards are one of the most common avian species on the planet, which is owed to the fact that they are able to adapt to environments disturbed by human activities, and are a common sight in many cities (Cramp and Simmons, 1977; Drilling et al., 2002). Mallards and other dabbling ducks are the natural reservoir for IAV, are known to harbour high prevalence of IAV in the wild, and may be implicated in the spread of highly pathogenic IAV (Latorre-Margalef et al., 2014; van Dijk et al., 2015; Verhagen et al., 2015). Indeed, the H1N1 IAV isolated in this study was more similar to viruses isolated in Asia than Europe suggesting long distance dispersal prior to circulation in this urban duck pond. This study was undertaken in 2014, prior to the influx of highly pathogenic H5N8 which were carried by apparently healthy birds (Verhagen et al., 2015). Given these urban Mallards harbour "Asian" IAV there is certainly concern for zoonotic spillover. Furthermore, it is not a stretch to imagine that Mallards may also be reservoirs and important in the spread and dynamics of other economically relevant RNA viruses such as CoV, APMV-1, and AstroV. Of all pathogens, RNA viruses are the most likely to be zoonotic (Woolhouse and Gowtage-Sequeria, 2005), and it is in environments where humans are in close proximity to a high density of birds that zoonotic spillover is most likely to occur. For example, live-bird markets are central in the transmission of avian influenza viruses from birds to humans (Wan et al., 2011). The role of urban ducks, given low virus prevalence, is uncertain, however, to better understand the zoonotic risk a better understanding of the RNA virus diversity and wildlife-pathogen dynamics in urban landscapes is crucial.

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Appendix A. Supplementary data

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