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Ecology of avian influenza viruses in a changing world

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Influenza A virus infections result in ∼500,000 human deaths per year and many more sublethal infections. Wild birds are recognized as the ancestral host of influenza A viruses, and avian viruses have contributed genetic material to most human viruses, including subtypes H5N1 and H1N1. Thus, influenza virus transmission in wild and domestic animals and humans is intimately connected. Here we review how anthropogenic change, including human population growth, land use, climate change, globalization of trade, agricultural intensification, and changes in vaccine technology may alter the evolution and transmission of influenza viruses. Evidence suggests that viral transmission in domestic poultry, spillover to other domestic animals, wild birds and humans, and the potential for subsequent pandemic spread, are all increasing. We highlight four areas in need of research: drivers of viral subtype dynamics; ecological and evolutionary determinants of transmissibility and virulence in birds and humans; the impact of changing land use and climate on hosts, viruses, and transmission; and the impact of influenza viruses on wild bird hosts, including their ability to migrate while shedding virus.

Keywords: H5N1 avian influenza; H1N1 swine flu; climate change; global warming; emerging infectious disease

Introduction

Emerging infectious diseases have been increasing in incidence and are a key threat to wildlife and human health.[1,2] Of the more than 335 human diseases that have emerged over the past six decades, influenza is one of the more prevalent, causing millions of severe illnesses and ∼500,000 deaths per year.[3] In addition to the disease burden caused each year by seasonal influenza there is a larger threat: the emergence and pandemic spread of a novel and virulent strain of influenza virus against which there is little or no immunity.

There have been three influenza pandemics in the 20th century, in 1918, 1957, and 1968, which resulted in approximately 50 million, 1 million, and 0.5 million deaths, respectively.[3] Recent concerns over an imminent influenza pandemic were focused on the H5N1 avian influenza virus that emerged in China in 1997,[4] primarily due to its high case fatality rate.[5] However, a novel strain of influenza containing genes from swine, avian, and human influenza viruses (subtype H1N1) emerged in Mexico in the spring of 2009 and has since spread worldwide.[6] Although H1N1 is the current focus of vaccine efforts,[7] concerns over the pandemic potential of H5N1 avian influenza remain high.

Wild and domestic birds are recognized as the reservoirs of most influenza A viruses.[8] Although the extent to which birds are involved in the emergence and global spread of novel, pandemic human strains remains debated,[9] even the most recent pandemic strain, H1N1, contains several segments that most likely originated in birds.[6] Thus, the dynamics of influenza infections among birds and mammals (including humans) are intimately linked.

Here, we review the ecology of avian influenza, focusing on the role of anthropogenic change in altering influenza transmission, evolution, and emergence. We begin by reviewing some features of the virus that make it so highly adaptable. Next, we discuss the role of the two most important groups of wild birds, order Anseriformes (ducks, geese, and swans) and order Charadriiformes (shorebirds and gulls) in influenza transmission and spread. We then discuss the implications of land use change, climate

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change, modernization of agriculture, new vaccine technology, and globalization on the transmission and prevalence of influenza viruses. We highlight the factors that have given rise to the most recent H5N1 and H1N1 influenza strains and their pandemic spread. Finally, we conclude by identifying four critical gaps in our knowledge about avian influenza ecology that, if better known, could improve our ability to predict (and even prevent) the emergence of the next pandemic influenza strain.

The virus

Influenza viruses are negative-sense single-stranded RNA viruses in the family Orthomyxoviridae. Their genome is composed of eight segments that, in total, code for 11 proteins, and have a total length of ~13.6 kb.[8] Two of these segments code for the surface proteins hemagglutinin (HA) and neuraminidase (NA), and these proteins are used in the subtyping of influenza viruses (i.e., H5N1). At present, 16 types of HA and 9 types of NA are recognized.[10] The HA protein is important in cell entry and interactions with the host immune system, whereas the NA protein catalyzes viral release from infected cells.

Three important traits make influenza viruses highly adaptable, able to evade host immune responses, and able to infect new host species.[8,11,12] First, the enzyme that catalyzes the replication of the RNA from the RNA template (RNA-dependent RNA polymerase) is error-prone. Second, there is a lack of error correction during replication. Finally, the structure of the influenza virus genome allows for exchange of entire segments between viruses coinfecting a cell, a process termed reassortment.

An important trait for the transmission and virulence of influenza viruses is their tissue tropism or infectivity of different tissue types in hosts. In birds, most influenza viruses primarily infect the cells of the intestinal, and to a lesser extent, respiratory tracts and are thus shed with feces, and respiratory secretions.[8] These viruses are generally thought to cause only mild illness in wild birds. Some strains, however, are able to invade and replicate in other cell types and organs, and as a result, may cause systemic and highly pathogenic infections. The current designation of an influenza virus as high or low pathogenicity is based on the severity of illness in poultry. For example, one index measures the morbidity and mortality of 6-week-old specific pathogen free chickens in the 10 days following intravenous injection of the virus.[13] Viruses that are highly pathogenic in chickens frequently (but not always; see Ref. 14) have a characteristic genetic alteration in the gene coding for hemagglutinin (e.g., a polybasic cleavage site in H5N1) compared to similar low pathogenicity forms.[15,16]

Influenza virus transmission is driven by interactions between the host community, the environment, and coevolution between the host and pathogen.[17] Anthropogenic environmental changes, including agricultural development, land use, globalization, and climate change have altered the ecology and evolution of influenza viruses and we explore their impact both individually and synergistically. If we can understand the ecology and evolution of influenza viruses in wild and domestic birds, this may enable us to identify the processes that contribute to emergence of the next pandemic human strain.[18,19]

The role of wild birds in influenza ecology

Wild birds have been found infected with the majority of the known subtypes of influenza A viruses.[20] Coinfections within individual birds of two viral subtypes enable reassortment of HA and NA genetic segments and have helped spawn the diversity of human and domestic animal strains.[17] Waterbirds, including primarily anseriforms (Order Anseriformes, including ducks, geese, and swans) and charadriiforms (Order Charadriiformes, including gulls, terns, sandpipers, plovers, etc.), are recognized as the natural reservoirs of influenza A viruses.[8,17,21] Infection prevalence in passerine (songbird) populations is usually much lower than that in waterbirds, suggesting that passerines are primarily spillover hosts (having been infected through contact with poultry or waterbirds). However, some peri-domestic species such as house sparrows (*Passer domesticus*) may be important in moving viruses between poultry farms.

Influenza in wild waterfowl

Influenza viruses circulate in many waterbirds, but are most prevalent in dabbling ducks (*Anas* spp.) and in particular, mallards (*Anas platyrhynchos*).[17] Nearly all of the 16 described HA and 9 NA antigenic subtypes have been found in dabbling ducks (Fig. 1).[10,17,22–24] Influenza prevalence among

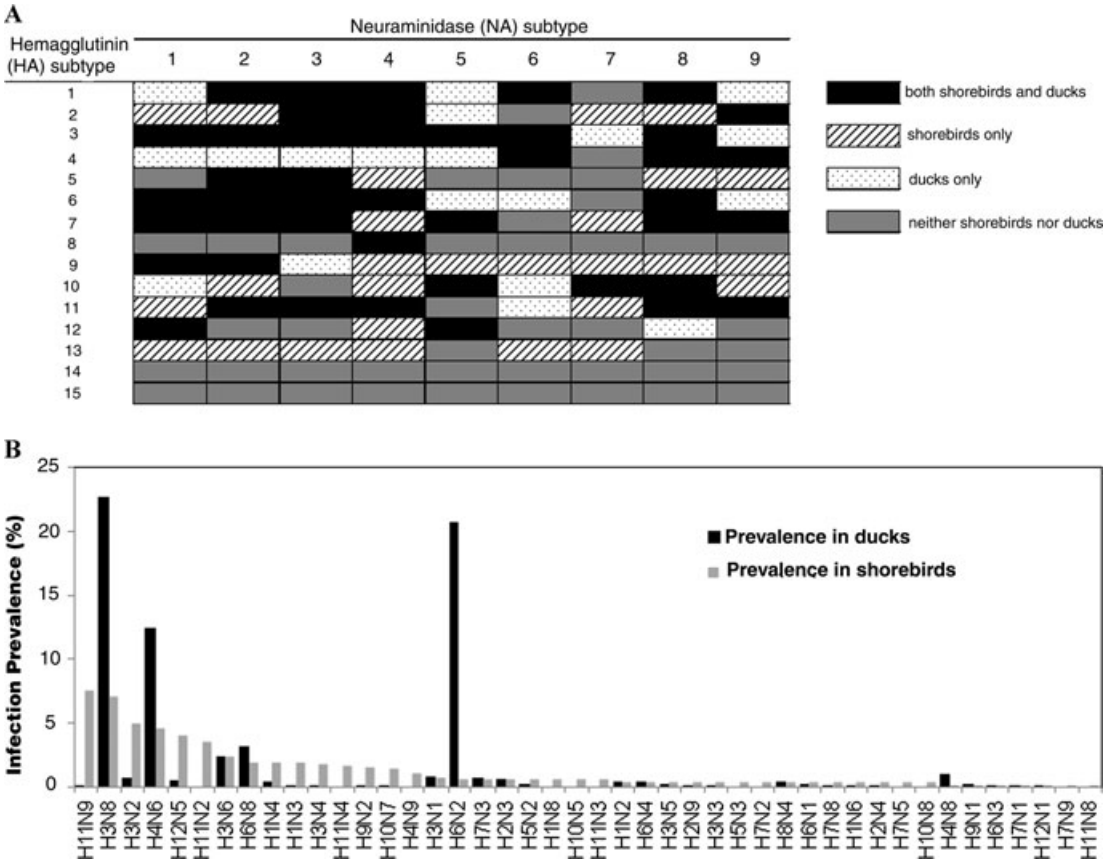

Figure 1. Presence and prevalence of influenza virus subtypes in shorebirds (charadriiforms) and ducks (anseriforms) from a 25-year study in North America.[22] (A) Presence and absence of viral subtypes in the two groups of birds. (B) Fraction of birds shedding virus of each of 44 influenza virus subtypes for shorebirds and ducks. Shorebirds were sampled during spring migration (May) in Delaware Bay, New Jersey, USA, whereas ducks were sampled in the late summer and fall (July–September) in Alberta, Canada.

mallards varies seasonally from <10% in the spring and summer to between 10% and 60% just before and during the autumn migration, and this pattern is seen in both Eurasia and North America.[17,22,24,25] Seasonal variation in infection prevalence is thought to be driven by the influx and aggregation of naïve juvenile birds following breeding and prior to (and during) migration,[8] and may also be influenced by environmental conditions that determine viral survival outside the host.[26]

Until the widespread emergence of H5N1 in 2002, influenza viruses were thought to cause little morbidity and mortality in wild birds. There was an early report of an influenza virus causing mortality in wild birds (an H5N3 virus in common terns [*Sterna hirundo*] in South Africa in 1961[27]), but few reports in the next 40 years. However, since 2002, H5N1 has caused mortality in dozens of species of birds from several different taxonomic orders including anseriforms.[28–33] In addition, recent evidence suggests that even low pathogenicity influenza viruses can cause illness in mallards and other species. In one study, infected mallards weighed almost 10% less than uninfected birds, and infected juveniles had increased staging times before migration in September.[23] In another study, Bewick's swans (*Cygnus columbianus bewickii*) infected with a low pathogenicity influenza virus traveled shorter distances and had reduced foraging/refueling rates.[34]

One continuing conundrum is that despite clear evidence of H5N1 being present in an area (e.g., the presence of H5N1-infected dead birds), it is rarely

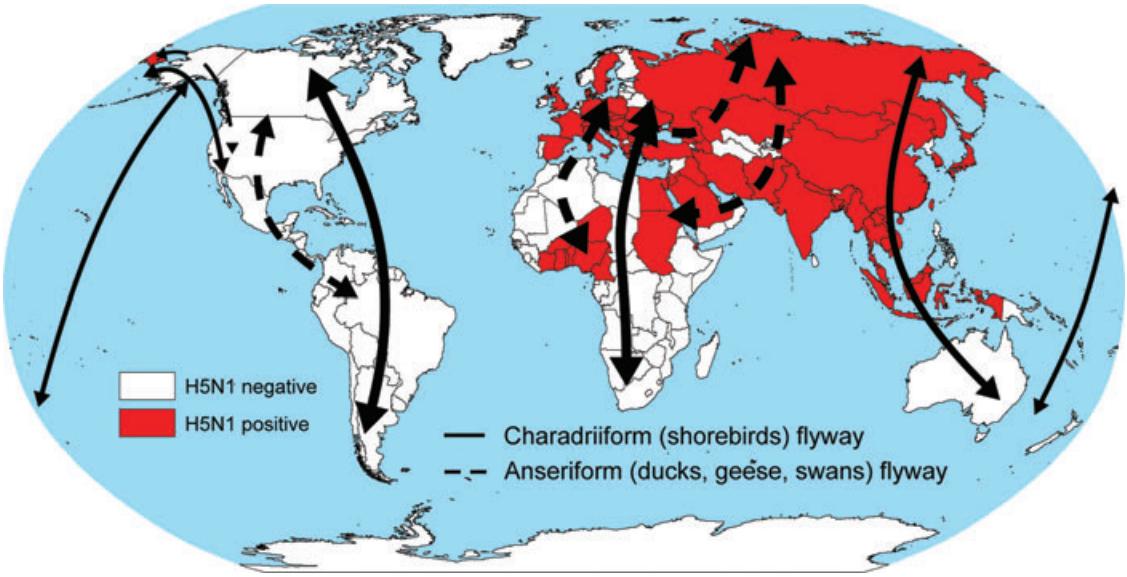

Figure 2. Intercontinental shorebird and Anseriform connections and H5N1 avian influenza distribution. Global map indicates countries (in red) that have had outbreaks of H5N1 avian influenza in poultry or wild birds between January 1, 2003 and August 15, 2009. Overlaid, in blue are links between breeding and wintering habitats of shorebirds (*solid lines;* Order charadriiforms, including gulls, plovers, sandpipers, etc.), and ducks, geese, and swans (*dashed lines;* Order anseriforms). Thickness of line indicates relative number of birds migrating between continents (*e.g.,* thousands of Anseriform birds migrate from Siberia to the Pacific coast of California, whereas millions of anseriforms migrate between Asia and Europe). Note also the distance each group migrates as indicated in the figure (shorebirds reach southern South America, South Africa, and Australia, none of which are reached by anseriforms from northern North America, Europe, or Asia). *Arrows* do not necessarily indicate actual flyway used by birds, but merely indicate connections between regions.

detected in living healthy wild birds.[35] This has led some to argue that the role of wild birds in the spread of H5N1 is relatively minor.[36,37] However, laboratory studies have shown that mallards and other species can remain healthy and shed virus following infection with H5N1.[32,38] In any case, the very low prevalence of virus in live, healthy birds has created challenges for H5N1 surveillance using these birds, and currently the most effective technique for detecting the presence of H5N1 appears to be monitoring for individual sick or dead birds.[33]

At root in this debate is the critical question: Can infected migratory birds fly significant distances while shedding highly pathogenic viruses? A direct experimental study would require a Biological Safety Level 3 laboratory equipped with a wind tunnel, and none of these yet exist. An alternative approach would be to use satellite-telemetry transmitters to track free-living H5N1-infected wild birds and recapture them to demonstrate continued viral shedding. However, because satellite-telemetry collars are expensive (several thousands of dollars per unit) and the prevalence of highly pathogenic H5N1 avian influenza in wild birds is frequently very low, even in outbreak areas (*e.g.,* 0.12%[39]) it would be very costly to ensure that infected birds were included in a sample. One strategy would be to develop rapid field tests for influenza infection (including subtyping) and use these to identify birds naturally infected with H5N1. These birds could then be fitted with a satellite transmitter. However, it is unclear whether it would be ethical to release birds known to be naturally infected with H5N1 and experimental infections in the field would certainly be considered unethical.

A related question is what role different taxa of birds play in the ecology of both high and low pathogenicity influenza viruses. Although dabbling ducks frequently show the highest prevalence of infection,[8,17] another group, charadriiforms are more

numerous, migrate longer distances, and also host a diverse set of influenza viruses.[20]

Influenza in shorebirds

Birds from the order Charadriiformes include shorebirds, gulls, and terns, and occur in large numbers on all continents.[40] The pattern of influenza infection in charadriiforms differs slightly from that in anseriforms. First, two hemagglutinin subtypes, H13 and H16, have almost exclusively been isolated from charadriiforms.[20,22,41] Second, at least in the Americas, shorebirds show peak infection prevalence in the late-spring and early-summer,[22] whereas ducks have higher prevalence of infection in the fall, just prior to migration toward the wintering grounds.[42] This pattern suggests that shorebirds may be important in the overwinter maintenance and long-term persistence of influenza viruses among wild birds, because they may serve to spread the virus to the northern breeding areas in spring.

Studies comparing patterns of avian influenza viral infection in charadriiforms and anseriforms have mostly emphasized differences in influenza patterns among these two groups.[20,22] Although important differences do exist, we reanalyzed a recently published large data set[22] and found that there were more similarities in viral subtype infections than would be expected by chance. A total of 63 HA–NA subtypes were found in a sampling of 13,466 North American ducks over 26 years and 71 subtypes were found in 4266 North American shorebirds over 16 years (Fig. 1A). We used a permutation test and found that the concordance of the presence and absence of HA–NA subtypes among ducks and shorebirds in North America was significantly higher than would be produced from a random distribution of HA and NA types present in each group ($P = 0.009$). Thus, if a subtype occurred in shorebirds in North America, then North American ducks were significantly more likely to carry that subtype, and *vice versa* (odds ratio 2.32, 95% CI 1.08–4.94). There is also a weak but significant correlation between the prevalence of influenza viruses on a serotype-by-serotype basis among shorebirds and ducks (Fig. 1B; Spearman's rho 0.21, $P = 0.02$). After excluding the serotypes for which the prevalence was zero in either shorebirds or ducks or both, the correlation was marginally nonsignificant (Spearman's rho 0.28, $P = 0.06$). In any case, the correlations were strongly influenced by a few points and may not be generalizable. In addition, it should be noted that the presence of a viral subtype in either or both groups was highly dynamic and may depend on the presence or absence of other subtypes due to cross immunity.[43] For example, one of the most common subtypes in ducks in the first half of this three-decade study (H6N2) disappeared from the sampled population and was not isolated for the rest of the study whereas others were only present afterward.[22]

The concordance of viral subtypes and prevalence of infection in ducks and shorebirds suggests several nonexclusive testable hypotheses involving hosts, viruses, and transmission ecology: (1) *host factors*: shorebirds and ducks share patterns of susceptibility and resistance to different subtypes, (2) *viral factors*: viral strain competition within hosts or the environment favors particular HA–NA subtypes that then infect both shorebird and duck populations independently and/or some HA–NA subtypes result in nonviable viruses, and (3) *transmission*: there is direct spillover of viruses from shorebirds to ducks and/or *vice versa*. Genome sequence analysis of viral subtypes that are common among ducks and shorebirds that live in, or transit, the same locations may be the most useful method for determining the extent of local spillover, but to date, few if any such detailed analyses have been performed.

Isolation of highly pathogenic influenza virus subtypes from charadriiform birds is generally a rare event. For example, out of 4623 confirmed highly pathogenic H5N1 avian influenza events reported from January 2004 through August 2009, 3048 were reported in domestic chickens, where surveillance is highest, and 588 in wild bird species, with most events being detections of single dead birds.[33] H5N1-infected charadriiforms (shorebirds and gulls) were only reported seven times (1.2% of wild bird detections). H5N1 has mostly been found in mallards, swans, geese, and other ducks (62% of wild bird detections or 384 events) and other species, including quail, buzzards, falcons, pigeons, guinea fowl, herons, grebes, crows, sparrows, and "unspecified birds" (39% or 234 events).[44] Although detection and reporting biases clearly exist (*e.g.,* swans are much larger than sandpipers and thus are more likely to be reported to health officials when they are sick or dead), it is less clear whether the low number of H5N1 events involving charadriiform birds is due solely to reporting biases or whether they are inherently less likely to become infected, get sick, or die

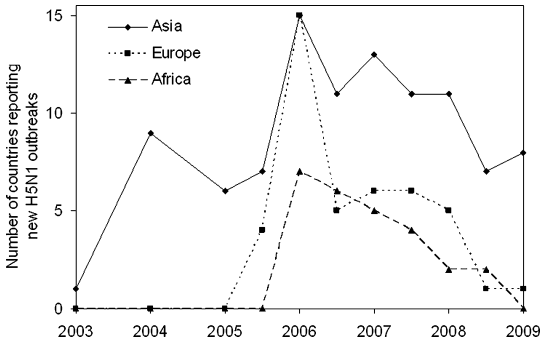

Figure 3. Temporal patterns of H5N1 outbreaks by continent. The number of countries in each of three continents reporting new H5N1 avian influenza outbreaks for each 6-month period from late 2003 through early 2009.

from this virus. Surveillance at sites along shorebird flyways, including birds traversing from Asia to New Zealand, Australia, or the West Coast of the United States, have found no H5N1 viruses and have documented very low prevalence of other viral subtypes in shorebirds, especially compared to ducks.[21,45–47]

Although H5N1 avian influenza can eventually be lethal to gulls,[50] the latency and shedding periods in shorebirds are only beginning to be studied. Recent work suggests that herring gulls can shed influenza virus for 1–5 days prior to the onset of any detectable clinical signs of illness.[52] Because some shorebirds can fly thousands of kilometers in just a few days,[51] the long-distance movement of viruses remains possible during the preclinical periods. Thus, even if shorebirds have lower prevalence of influenza infection during migration than ducks, the potential global distribution of viruses, and the flow of viral genes, could be affected by shorebird movements.[11,53] Further research documenting the clinical course of influenza infection in charadriiforms, and their capacity to migrate while infected, are needed.

Even if rare, the consequences of shorebird-mediated spread of influenza could be important. This is because shorebirds migrate over much greater distances than anseriforms (ducks, geese, and swans), and connect several geographic regions that anseriforms do not (Fig. 2). For example, large numbers of shorebirds regularly migrate from Europe to South Africa, from Asia to Australia, and from eastern Siberia to the lower 48 states of the United States. In contrast, many fewer anseriforms, or none at all, migrate between these regions.[30,48] Phylogenetic analyses of viruses isolated from shorebirds in different continents have been useful in detecting evidence of these movements.[49] In addition, contact and aggregation patterns among charadriiforms, ducks, and domestic poultry are poorly studied despite their importance for viral spillover. These contact patterns will be significantly affected the distribution and quality of wetland habitats, which have been severely altered by anthropogenic environmental change.

Wetlands, land use change, and the transmission of avian influenza

Wetlands provide critical habitat for wild waterfowl as well as ecosystem services such as sediment trapping, ground water purification, and water velocity reduction during floods. Yet, over the last several centuries, a third of all wetlands have been dammed, drained, and used for development, agriculture, golf courses, and domestic water use.[54–56] Some studies also suggest that wetland habitats are disappearing as temperatures climb due to decreased inflow and increased evaporation as glaciers and snow-packs shrink.[57] Wetland loss has been highly heterogeneous such that some areas, such as California, have lost over 90% of their wetlands, whereas other less accessible areas, such as Alaska, have lost less than 5%.[58] Land use change, coupled with global climate change and increased demand for freshwater by humans, is expected to significantly decrease the global availability of natural wetland habitat for wild waterfowl in the future.[55]

Loss of wetlands has had variable effects on wild waterfowl populations. Some duck species, such as northern pintails and in North America, have suffered population declines in the last several decades linked to habitat loss.[59,60] Other waterfowl, including some species of geese and swans, have adapted to human-altered landscapes and have experienced spectacular population increases over the last few decades. Their increases are likely in part due to food subsidies provided by nitrogen and calcium rich croplands that are important in reproduction.[61,62]

In many countries, there has also been a significant shift toward agricultural intensification. An example of this is the decrease in the agricultural practice of "summerfallow," where land was

traditionally tilled but left uncropped for 1 year to allow for moisture accumulation, nitrogen release, and weed-control benefits. The shift toward continuous cropping is one of the most economically and environmentally significant land use changes in Canadian prairies in recent decades.[63] Waterfowl nests, including those of northern pintails (*Anas acuta*), in agricultural land are often destroyed by the modern practice of continuous cultivation.[59]

The consequences of these changes in land use and wild waterfowl populations for avian influenza ecology are only beginning to be explored. Land use change that leads to higher waterfowl densities, stress, or proximity to domestic birds, will likely lead to increased influenza transmission within flocks and increased cross-species transmission. For example, in Thailand irrigation has enabled year-round or "second harvest" rice cropping that supports higher densities of domesticated free-grazing duck populations that rely on postharvest rice fields for feed.[64] In these areas, highly pathogenic avian influenza H5N1 outbreaks in domestic poultry are frequent, suggesting a possible causal link.[65]

A complicating issue is that decreasing wetlands may have a dynamic effect on influenza transmission. Initially, aggregations of waterfowl may be increased due to crowding at breeding, wintering, and stopover areas, which may also lead to increased contact rates between wild and domestic birds. In the longer term, waterbird populations dependent on wetlands are likely to decrease, most likely with a shift to more human-commensal species (e.g., Canada geese, *Branta canadensis*). The critical question is whether the species that remain are effective amplification hosts for influenza viruses.[66] At present, the consequences of longer-term ecological changes on influenza transmission are not well known, and will be dependent on the interactions among hosts, viruses, and the environment, including climate change.

Climate change and avian influenza

Evidence of climate change now includes rapid temperature increases near the poles, increased rainfall in some areas (*e.g.,* eastern parts of North and South America, northern Europe, and northern and central Asia) and drought in others (*e.g.,* the Sahel, the Mediterranean, southern Africa, and parts of southern Asia).[57,67] These changes in rainfall alter the distribution, abundance, and quality of wetlands and can impact waterfowl populations. For example, higher precipitation and warmer temperatures were correlated with higher abundance of waterfowl in Canada,[68] and the size of duck populations in North America was tightly coupled to the availability of wetland (pond) habitat from the 1960s until the 1980s (but less so afterward).[59,60] Climate-associated changes in waterbird abundance will likely be geographically variable as well as species dependent. For some species, climate change may lead to range shifts,[69–71] whereas for some high arctic breeders, climate change may lead to an almost complete loss of habitat.[72] For all species, substantial uncertainty exists because there will likely be interactions between climate and other factors, such as land use change, that influence overall abundance patterns.

Climate change also appears to be altering migration patterns of many species of birds, but with differential effects. Some long-distance migrant birds show earlier spring migration,[73] whereas others do not.[74] Climate-associated changes in the distribution, composition, and migratory behavior of wild bird hosts may cause important changes in avian influenza epidemiology. However, geographically specific directional predictions will require a knowledge of the behavioral (e.g., contact) patterns of species, as well as their susceptibility and infectiousness for influenza viruses, which are at present largely unknown.[75]

In addition to impacts on avian hosts, climate change may have important impacts on the epidemiology of avian influenza through viral survival in the environment. Influenza virus survival decreased in the laboratory with increased water temperature, more acidic conditions (lower pH), and higher salinities.[26,76] All of these changes are expected to occur in most freshwater ecosystems with future climate warming scenarios,[55] which may decrease the survival of influenza viruses in the aquatic reservoir and thereby decrease transmission. Predicting net impacts of climate change will require models and empirical investigation of how decreased viral survival in the aquatic reservoir might interact with other factors determining influenza transmission, such as smaller, shallower wetlands, and increased crowding, stress, and contact rates among migratory species and between wild and domestic birds.

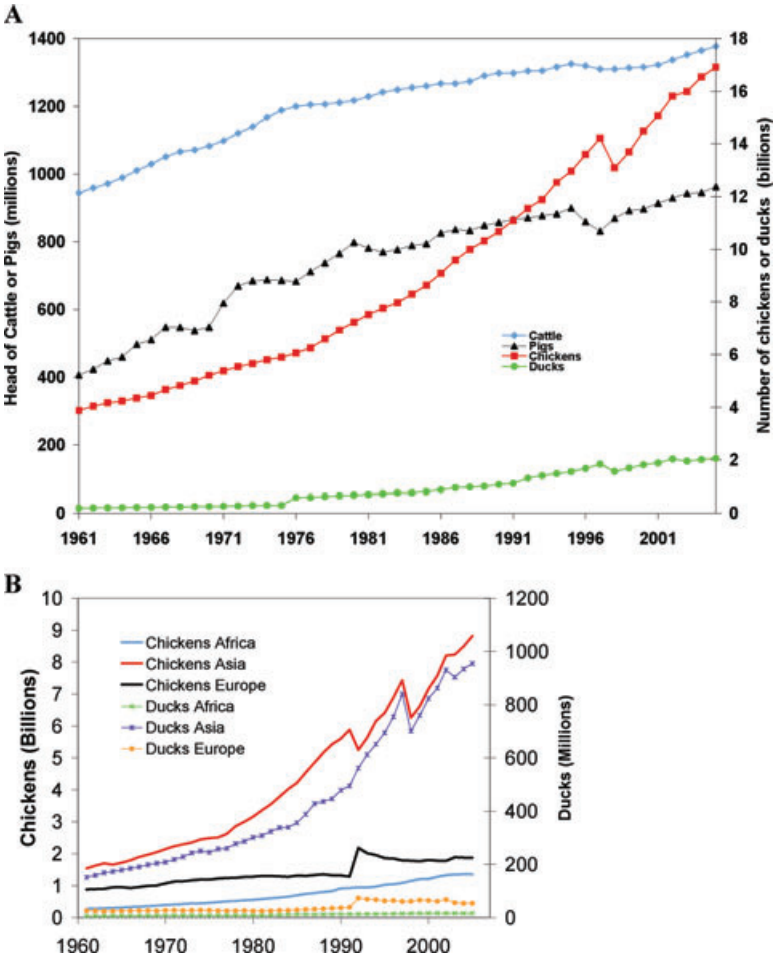

Figure 4. Production of four different stocks, from 1961 to 2005 based on FAO data. (**A**) Global production of poultry (*ducks* and *chickens*), *cattle,* and *swine*. (**B**) Production of ducks and chickens by geographic region over the same period.

Agriculture, trade, and influenza in the 21st century

Between 1960 and 2002, human consumption of chicken more than tripled, whereas production of other livestock increased, but more slowly (Fig. 4A).[77] Although there is enormous variation in the methods of poultry production, the global trend is toward an increase in the size of farms. Although the number of farms has decreased by 50%, production has gone up over 500%,[77] and these production increases are even stronger for Asia than other regions (Fig. 4B).

The development of large-scale commercial agriculture has enhanced the potential for epidemic transmission and evolution of influenza viruses. An average commercial broiler facility can generate about 500,000 birds per year (2.6 million pounds of meat).[78] To achieve these high rates of growth, substantial genetic selection for improved growth and feed conversion on poultry breeds has occurred and has resulted in reduced genetic diversity of domestic poultry. Animals kept in high concentrations typical of commercial production facilities also experience crowding stress, which might make them more susceptible to pathogens such as coccidia, Marek's disease virus, Newcastle disease virus, and influenza viruses. Finally, although biosecurity within these farms is generally high, recent spillover events of H5N1 between wild birds and poultry[30] indicate

that gaps still exist, and the large size of poultry farms makes subsequent spread of virus potentially explosive.

The stocking densities in large commercial farms also facilitate rapid and efficient transmission of highly virulent viruses such as H5N1 that might otherwise kill their hosts before being transmitted (*e.g.,* H5N1 frequently kills chickens in <48 h[82]). In addition, previous work has shown that repeatedly passing a low pathogenicitiy virus among susceptible chickens can result in the evolution of a highly pathogenic strain.[81] This has led some to speculate that the highly virulent H5N1 strain was able to emerge because of the intensification of poultry production, but at present, there is little direct evidence for this having occurred.

Small family farms with free range ducks or chickens also present risks for the transmission of avian influenza viruses.[64,65] These farms have little or no biosecurity, and the domestic birds are frequently free to mix with wild species. This mixing may have facilitated the spread of H5N1 in Russia, China, and elsewhere during the intercontinental spread of H5N1 from 2004 to 2006.[30,83] Another key feature of backyard poultry is the frequent occurrence of a staggered age structure of birds (e.g., chicks, poults, and adults), which can support persistent enzootic virus transmission that is less likely in "all in, all out" procedures in large-scale poultry farms. Finally, in many developing countries there is a high contact rate between backyard poultry animals and humans, including a common practice of keeping birds inside homes during the night (e.g., in baskets beneath the owners' beds; Kilpatrick and Vandegrift, personal observation).

Live bird markets also play an integral role in the dynamics of influenza virus transmission and evolution. Live animal markets have a long history in Asian and other cultures, partly because of a lack of refrigeration that make purchasing a whole live animal the only way to assure poultry product freshness. Live bird markets have been implicated in a number of avian influenza outbreaks and in facilitating enzootic influenza virus transmission worldwide,[81] including H5N1 in Hong Kong[28] and in other parts of Asia.[84] Markets can also be hotspots for viral evolution because they bring together different species of animals from different geographical areas into an unsanitary environment and this can facilitate reassortment between viruses.[85]

Research on viruses circulating in live bird markets has helped us learn about the processes of viral evolution and transmission.[39,86,87] For example, domestic and wild bird surveillance and phylogenetic reconstruction resulted in the identification of H5N1 as a pandemic threat as early as 2002, when its distribution was limited to China, and only a few humans had been infected.[88] Actions taken to prevent transmission of this and other influenza viruses in markets included separation of different types of poultry in markets and a "day of rest," to allow for cleaning of cages and elimination of viruses and other pathogens.[89,90] Unfortunately, preventative measures were unsuccessful in entirely stamping out the H5N1 virus, and it reemerged in 2003 and spread throughout Asia, Europe, and Africa over the next 4 years (Figs. 2 and 3).[30]

The emergence of H5N1 has led to four questions being asked of new avian influenza virus isolates acquired during market surveillance: What are the virus' effects upon different hosts (i.e., how pathogenic is it)? Can it infect humans? How transmissible is it between birds and between humans? And, is it resistant to antiviral drugs? Tools to answer these questions cheaply and quickly are being developed, and should enable scientists and public health workers to take a more active role in preventing the emergence of influenza viruses in the future.

Globalization of trade

Over the past decade, European Union countries are estimated to have exported over 18 million live poultry and over 750,000 live swine to North America.[77] Although biosecurity, quarantine, and testing procedures greatly reduce the risk of viral spread, occasionally, trade of infected poultry occurs before a local epidemic is detected. Because of the large numbers of birds involved, poultry trade can enable viruses to spread rapidly, as occurred in the H7N2 virus outbreaks in Pennsylvania in 1996–1998.[91]

Trade in poultry from and between developing countries may carry a larger risk, per chicken, of viral spread, due to infrequent testing or quarantine, and less regulated trade. In addition, government practices of reimbursing only a fraction of the market value of birds to farmers during culling of infected and/or sick animals sometimes encourages rapid and often illegal selling of birds before they die or are seized and culled. Studies to objectively assess the risks of different farming strategies for

the perpetuation and spread of influenza are just beginning, and must account for complex political, social, and cultural issues.

Additional pathways for the spread of influenza viruses and other pathogens are the legal and illegal trade in wild animals (including birds) and the illegal trade of poultry and poultry products. Wild bird trade is a globally important phenomenon which carries the risk of introducing several pathogens including influenza, West Nile virus, and others.[30,92,93] The risks associated with these animals are highly variable, depending on whether the animals are captive-bred or wild-caught, and whether they are moved legally or illegally. Illegally traded animals bypass any testing and quarantine requirements that may be in place, and thus are a significant threat.[94] Indeed, on two separate occasions, illegally traded wildlife with active H5N1 infections entered Europe; however, they were intercepted before mixing with and potentially spreading the virus to local birds.[30]

Actions to reduce the spread of avian influenza and other pathogens are beginning to be enacted across the spectrum of poultry production. Live animal markets, which are known to be high-risk areas for the transmission and spread of influenza viruses and other pathogens, are being increasingly regulated, or closed altogether.[84] International trade of live poultry has also been increasingly regulated or banned from some countries.[95] Evidence of the impact of regulation on markets exists in Vietnam, where regulation has led to a significant decline in the number of ornamental bird markets.[96] One final measure that has received substantial attention for controlling H5N1 in poultry flocks is vaccination.

Vaccination and control measures for influenza

Two primary strategies currently exist for influenza control: vaccination and depopulation. The second is clearly costly but is frequently the method of choice when epidemics of highly pathogenic influenza occur within poultry houses.[97] Vaccination, on the other hand, is often undertaken as a preventative measure, although it has also been shown to be effective during an outbreak of H5N1.[98] Most vaccines reduce both susceptibility and infectiousness, and thus have dual benefits. Several countries including China, Vietnam, Pakistan, Indonesia, Thailand, and Egypt have or are currently executing costly vaccination programs for live birds because of fears that H5N1 viruses have become enzootic in Southeast Asia.[5,39] Two key challenges for influenza virus control using vaccines are (1) high costs associated with current vaccine production techniques that require culturing virus in embryonated chicken eggs and (2) maintaining an effective vaccine against a fast-mutating virus. Innovative techniques, including reverse genetics and chimeric constructs, are being developed to produce cheaper vaccines more rapidly, as well as vaccines against multiple pathogens, such as influenza and Newcastle disease.[99]

One important issue with vaccination is that it may promote enzootic transmission and spread by masking the presence of highly pathogenic viruses such as H5N1. These viruses would normally be detected by higher than normal mortality during production or shipment of poultry, but not in vaccine-protected poultry which sometimes still shed virus.[100] In some cases, the use of nonsterilizing vaccines may also facilitate evolution of increased virulence.[101] Overall, vaccination is a useful tool in influenza control, but costs are substantial, especially given the short lifespan of the commercial birds, and it brings with it the additional risks of enabling silent transmission and encouraging evolution of the virus. In humans, by contrast, vaccination is one of the primary influenza control measures, and the global spread of H1N1 influenza has highlighted the need for rapid production effective vaccines against novel strains.

Globalization, travel, and the pandemic spread of H1N1 influenza

Globalization has changed the landscape within which viruses such as influenza and SARS are maintained and transmitted, and thus has enabled rapid, unprecedented rates of disease spread.[6,30,102,103] In just over a month, a novel strain of H1N1 influenza spread throughout most of the United States and to 23 countries, including New Zealand, almost 15,000 km away from the virus' initial detection in Mexico. The number of early cases of H1N1 influenza in each country fit well with the volume of airline travel arriving from Mexico, providing both a link and perhaps a predictive tool for future spread of novel viruses.[6,104]

Air travel has not only increased global connectivity among humans, but it has done the same for livestock, including poultry, and their associated viruses. The viral strain involved in the most recent H1N1 influenza pandemic is a triple-reassortant, with gene segments from humans, birds, and swine.[105] It is well known that swine are particularly good mixing vessels for influenza viruses because they have cellular receptors in their respiratory tract that both avian and human influenza viruses can bind to.[81,106,107] It is impossible to determine where or in which hosts the mixing of viruses occurred because the virus has been evolving separately from its closest known relative for approximately 10 years. However, abundant evidence exists of intercontinental trade in poultry and swine, and this trade could have facilitated strain mixing and reassortment.[30,104]

The greatest future threat to human health from an influenza virus comes from a virus to which there is no preexisting immunity in the human population, one which has the transmissibility of seasonal flu and the virulence of some H5N1 viruses which can have a case-fatality rate greater than 50%.[108] The estimated economic impact of an introduction of a virus with these characteristics in the United States is between $71.3 and $166.5 billion, not including disruptions to commerce and society.[109]

Critical gaps in our knowledge of avian influenza

There are four areas of influenza virus ecology and evolution that are in urgent need of attention.

1. *Drivers of viral subtype dynamics in birds.* Although it is now relatively straightforward to isolate and determine the subtype of viruses from wild and domestic birds, we understand very little about the selective pressures on different subtypes, the degree of cross-immunity among subtypes, and the dynamics of coinfection of two or more subtypes within a single individual. A laboratory experimental infection studies with sequential infection of different viral subtypes would be a significant first step in increasing our knowledge. This study should characterize the total quantity of viruses shed (shedding period, fecal volume/day, viral titers in feces, shedding from the trachea, etc.) as well as host morbidity and mortality.
2. *Ecological and evolutionary determinants of transmissibility and virulence in wild birds and humans, and their interplay.* Evidence is accumulating that agricultural environments facilitate the evolution of virulent strains that kill poultry in just a few days. However, the effects of these environments on transmissibility (shedding volume, infectious dose, and contact rates between animals) are poorly known, making it difficult to develop nonvaccine strategies for retarding evolution of highly pathogenic strains. At least as important, and much more poorly known, are the ecological factors (especially anthropogenic changes currently underway) that influence viral evolution in wild birds. Finally, a key unanswered question is: to what extent does selection for virulence and transmissibility in avian hosts impact the likelihood of a pandemic strain emerging in humans?[110] For example, what traits have enabled the H5N1 virus to have such an extraordinarily broad host range that it can infect poultry, nearly all wild birds studied, and several orders of mammals, including humans?[18] All of these questions will require understanding the importance of genetic drift, reassortment, and coinfection in "mixing vessels" (animals that can be infected with avian and human strains such as swine) in influenza evolution.[81,106,107]
3. *Impacts of changing land use and climate on hosts, viruses, and transmission.* Many anthropogenic changes are relatively well described, such as changes in the extent of wetlands. On the other hand, the consequences of these changes, and fine scale patterns of other anthropogenic disturbances such as climate change on host population dynamics, aggregation, and migration patterns are unknown. When these impacts are better known, models could be integrated with data on viral survival in the environment[26,76] and data from the host-pathogen studies just described, to predict the impact of these changes on influenza transmission.
4. *The impact of influenza viruses on wild bird hosts, including birds' ability to migrate while infected and shedding virus.* Like many wildlife

diseases, the impact of influenza viruses on wild animal host survival, reproduction, and behavior are almost completely unknown, and these impacts have important conservation and management implications. One key question is: Are some wild birds able to migrate while infected and shedding virus? Available evidence suggests that infection with some strains influences the migratory behavior of some species. However, field studies have so far been limited to low pathogenicity strains and have examined too few species to produce recommendations that could be used to decrease the spread of highly pathogenic subtypes such as H5N1. For example, if carrier species could be identified, preventing contact between this species and domestic animals could reduce the spread of influenza.

At present, most research on influenza has been devoted to the development of vaccines for humans and poultry. In particular, the top priority has been development of new vaccine technologies that can be used to produce large quantities (i.e., hundreds of millions of doses) of vaccine in a short period of time. However, without additional understanding of the impacts of vaccination on viral evolution, these strategies, even under the best case scenario, will only slow down or delay epidemics. In the worst case, they may even facilitate the evolution of increased virulence.[111] Clearly, understanding the ecological and evolutionary aspects of transmission, including the environment of a changing world, are critical to minimizing the impacts of influenza on wildlife and public health.

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Conflicts of interest

The authors declare no conflicts of interest; NIH funded.

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