Incursions of Highly Pathogenic Avian Influenza Viruses into Japan: A Comprehensive Analysis with Special Reference to Virus Perpetuation within Domestic and Wild Waterfowl in Continental East Asia and Siberia

Dany Shoham[1,2]*, Hakim Hakimullah[1,3] and Kazuaki Takehara[1]

[1]*Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu- shi, Tokyo, Japan*
[2]*Bar-Ilan University, Begin-Sadat Center for Strategic Studies, Ramat Gan, Israel*
[3]*Faculty of Veterinary Science, Kabul University, Jamal mena, Kabul, Afghanistan*

*Corresponding Author: Dany Shoham, Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu- shi, Tokyo, Japan and Bar-Ilan University, Begin-Sadat Center for Strategic Studies, Ramat Gan, Israel.

Abstract

Highly pathogenic avian influenza viruses (HPAIVs) constitute an important category of pathogens, in terms of both animal health and public health. Alongside their devastating effects on the poultry industry, they virulently afflict humans, and, moreover, are often regarded to potentially be an imminent precursor of future pandemic strains. This applies particularly for East Asia.

Japan is appreciably vulnerable to incursions of HPAIVs, due to its ecogeographic position in relation to China, Mongolia and Siberia, where such viruses are locally entrenched and are liable to be carried by subclinically infected waterfowl migrating to Japan. Nevertheless, although the first HPAIV ever isolated in Asia appeared in Japan in 1925 already (an H7N7 subtype virus), only numbered additional occurrences of HPAIVs were recorded in Japan since then, mainly including six primary introduction events from 2003 to 2017, with no human cases. The sharp contrast marking the interface between Japan and nearby continental Asia in that respect has comprehensively been explored in the present study. The presented data and analyses highlight in detail distinctly broad ecoepidemiological and ecophylogeographic machineries. Those machineries are propelled by intensive interfaces that exist between continental Asia and Japan, and yet accentuate meaningful differences between those two regions, in regard to the generation, occurrence and persistence of HPAIVs. The complicated virological, pathobiological, and evolutionary dynamics of HPAIVs across the two regions is concretely demonstrated. The phylogeographic and phylochronological profiles constructed thereby underscore a coherent evolutionary apparatus that has direct interactions with Japan, and bears significant epidemiological consequences, in terms of animal and public health. Secondarily, but largely extended spatially, phylogenetic affinities emerge among Japanese, European, American and Oceanian HPAIVs, adding a further important dimension.

Keywords: Highly Pathogenic Avian Influenza Viruses; Virus Perpetuation; Domestic Waterfowl; Wild Waterfowl; Japan

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INTRODUCTION

Unlike most countries in Asia, Japan rarely experienced the presence and impact of potent viruses such as foot and mouth disease, rabies and highly pathogenic avian influenza (HPAI), in spite of its close proximity to continental Asia. Foot and mouth disease virus invaded Japan lastly in 2010, after ten years of absence. Exhibiting high homology to serotype O viruses isolated in Hong Kong, Korea, and Russia in 2010, the causative agent was regarded to may have entered Japan through the movement of people or commodities from those countries [1]. Further, Japan is one of the few rabies-free countries worldwide. Although 3 imported cases of human rabies were seen in 1970 and 2006, no other cases have been reported for ≈50 years. The elimination of rabies in Japan is attributed to not only its geographic isolation, but also to highly effective prevention and control measures [2].

Avian influenza viruses (AIVs) are enormously more mobile, nevertheless - due to their conveyance by migratory birds all across the globe - and therefore, analyzing their ecoepidemiological dynamics and phylogenetic courses is rather more complex and challenging. Comprising 16 HA antigenic subtypes and 9 NA antigenic subtypes, they are basically benign waterborne enteroviruses of a vast variety of wild birds, while two HA subtypes - H5 and H7 - at times transition into HPAIV by certain mutations (as necessary, yet often insufficient conditions), and can consequently infect and cause heavy looses of chickens and turkeys. HPAIVs are common in China, Mongolia and Siberia, three regions that represent prime nidi of HPAIVs, worldwide.

Japan constitutes a condensed group of islands located closely to continental Asia and comprising the eastern edge of Asia, right before the Pacific Ocean; it is hence the final stop of numerous migratory aquatic birds - the principal hosts of AIVs - that come to Japan from northwestern, western and southwestern territories. During the recent decade, it has been fully evidenced that various waterfowl are capable of carrying HPAIVs over long distances during their migrations, in spite of the fact that the very same viruses are lethal to chickens, turkeys and many wild avian species. Most probably, migratory waterfowl are the in effect main spreaders of the HPAIVs worldwide, particularly across East Asia and Siberia. The impact brought about by those viruses towards poultry farming is colossally harmful, and secondarily poses a sever public health menace as well, if not a potential for a shift into a pandemic strain.

Nevertheless, Japan experienced relatively few introductions of HPAIVs, with none becoming endemic, and with no human cases, while in nearby China, as well as in additional countries of East Asia, various HPAIVs are for long - and in all likelihood irreversibly - endemic. Endemicity in Siberia too is an actual, resultful possibility, as discussed below. Moreover, China is the chief melting pot of AIVs, including HPAIVs, and the most consequential generator and springboard of new HPAIVs that transfer to many countries, including Japan. Thus, the typical endemicity marking China is meaningful both in providing a constant reservoir of HPAIVs from which the viruses are conveyed to Japan by migratory waterfowl, on the one hand; and in underscoring Japan as a distinct 'antithesis', in that such viruses never became endemic in Japan during the last 20 years, on the other hand. It is therefore a notable challenge to thoroughly look into this ostensible equivocality, both scientifically and practically.

The natural ecogeographic system composed of Japan, China and Siberia, plus South Korea (the closest land to Japan, regardless of Sakhalin) and Mongolia (a prime natural nidus of HPAIVs) propels every autumn an influx of waterfowl-carried AIVs into Japan, which might always include HPAIVs, as a principle. North-to-south movements from east Siberia to Japan, and south-to-north movements from Oceania to Japan might be important too. Collectively, it means that there is a nearly constant possibility for incursions of HPAIVs into Japan, which not necessarily would be detected, unless infection of poultry occurs.

Very close to, yet isolated from continental Asia, with but a small portion of waterfowl within its poultry farming, and strict, fully implemented regulations for coping with HPAIV events, Japan represents a sharp contrast to China, Vietnam, Thailand and Cambodia within that context. The difference is accentuated in relation to Indonesia as well - another group of islands, located closely to continental Asia, which is endemically afflicted by HPAIVs, already for years. Broilers comprise over 90% of Japan's domestic poultry production, with spent hens comprising most of the remainder. Very few other poultry species are produced commercially, with the exception of a limited production of quail for eggs. Ducks and geese are nearly negligible.

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While the introductions of HPAIVs into Japan by migrating birds are obviously unpreventable, the longest period of persistence inside Japan of the detected HPAIVs was of 144 days (as detailed below), which is a marked duration. Throughout such a period of time, HPAIVs may basically be naturally attenuated, or, by contrast, persist and thereupon derive further, new HPAIV genotypes. At any rate, the factors facilitating persistence and disappearance of HPAIV are to be elucidated in the present study, with special reference to ongoing perpetuation of HPAIVs in continental Asia.

All in all, a unique ecoepidemiological setup is noticeable, accounted for by the particular interface that exists between Japan and continental Asia, in terms of a tangible, steady HPAIV threat posed by continental Asia, which materialized in Japan but seldom, though. This analysis aims, then, to comprehensively investigate the events of HPAIV invasions of and spread in Japan, thoroughly assess those well-defined events, and thereby intactly figure out broad, coherent ecophylogeographic and phylogenetic perspectives in regard to the underlying mechanisms and dynamics marking this remarkable system.

OCCURRENCES OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES (HPAIVs) IN JAPAN

Since 1925, Japan experienced 7 introduction events of HPAIVs, which included 4 antigenic subtypes, namely H7N7, H5N1, H5N8 and H5N6. An introduction event is defined here as one during which HPAIVs were continuously present in Japan. Secondary HPAIV introductions could have taken place during a given period of an introduction event. Following are epidemiological, phylogenetic and spatiotemporal data, analyses and observations regarding the various introduction events that underlay HPAI occurrences in Japan, allover. During 2014-2015 there were two separate introduction events, yet they are presented below, just textually, as one introduction event, due to their temporal proximity, the fact that they represent the only occurrences of the H5N8 HPAIV subtype in Japan, and the affinities displayed among the interrelated viruses. Each introduction event (except for the earliest, less documented 1925-1926 introduction event, and the quite fresh 2016-2017 introduction) is orderly presented and discussed along the following aspects:

- Initial indication
- Involvement of poultry
- Involvement of wild birds
- Contemporaneous and proximal occurrences of HPAI in neighboring countries
- Genomic characteristics of the viruses isolated in Japan
- Extraneous origin and spread in Japan.

Another episode, in which a H7N7 HPAIV was isolated from a cat, merely, happened in Japan in 1942, and is mentioned below. Also, a case of duck meat imported from China to Japan, and found infected with H5N1 HPAIV, has been reported in 2003 [3], irrespective of the introduction event that took place in that year, as discussed below.

1925-1926 - H7N7

Outbreaks of what was then termed 'fowl plague' (later on termed HPAI) occurred in several parts of Japan during 1925-1926, with symptoms typically including sever hemorrhagic lesions and high fatality, caused by a virus that was isolated in Chiba prefecture (Chiba strain), and thereafter identified as A/chicken/Japan/1925 H7N7 [4].

This epizootic yielded, thus, the first influenza virus ever isolated in Asia. While data pertaining to HPAIVs that emerged and circulated in East Asia since 1996 are available and detailed, the outstanding occurrence of HPAIV in 1925 in Japan was preceded by only two virologically featured influenza A virus (IAV) occurrences, worldwide: an H7N7 HPAIV that was isolated in Italy from chickens in 1902 (the first influenza virus ever isolated, globally); and the 1918 pandemic virus. Curious in themselves, certainly, those two earlier occurrences cannot provide, nevertheless, a concrete clue as to the mode underlying the appearance of the H7N7 HPAIV in 1925 in Japan. The latter either came into being in continental Asia and subsequently transferred to Japan, or formed in Japan consequent to a local transition

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from a LPAI H7 virus into a HPAI one. Noticeably, though, from 1902 to 1952 only H7 HPAI viruses were isolated in Europe, and none in continental Asia, but it would be implausible to conclude that the HPAIV occurrence in Japan in 1925 was the only one in Asia during that period. On the contrary, it may be conceived that a primal natural nidus of avian-equine H7N7 viruses prevailed throughout that period in Mongolia/Southern Siberia (sustained foremost by wild waterfowl and wild horses), compatibly with the particular features of that geographic belt, its fundamental role in the ecology of IAVs at large (as evidenced during the recent decades and detailed below), and comprehensive findings regarding the ancient phylogenetic avian-equine position of IAVs on the whole [5]. Horses in general, both wild and domestic, were much more prevalent during that time then today. Although far-reaching changes took place across East Asia since 1925, in many senses, it seems that the very fundamental AIV ecophylogenetic machinery still operates therein today, while H5 - rather than H7 - HPAIVs prevail, nowadays, and the influenza viral input generated by horses altogether - H7N7 and H3N8 - is considerably diminished, because during the 20th century the populations of horses, both wild and domestic, dramatically decreased in Asia. Thus, in terms of documented information, it is of note that the H7 and H5 viruses circulating in domestic and wild birds throughout Asia did not transit from LP to HP since the 1925-1926 HPAI epizootic in Japan, until 1994 (when a HPAI H7 virus emerged in Pakistan), while shortly afterwards, HPAI H5 viruses emerged in China, from 1996 onwards. The shift taking place thereafter in continental East Asia, namely the unprecedented proliferation of HPAI H5 viruses, was tremendous, and appreciably affected Japan since 2003. It is nevertheless plausible that undocumented occurrences of HPAIVs in Asia between 1927 and 1993 did take place; the mentioned isolation of another H7N7 HPAIV in Japan in 1942, from a cat, constitutes an indirect evidence in that respect.

2003-2004 - H5N1

Initial indication: Mortality of chickens was observed on the 28 December 2003 in Yamaguchi prefecture, leading to the ever first isolation of an H5N1 HPAIV in Japan. The source of this information, as well as of similar data presented hereafter, is the OIE Update on HPAI in Animals [6].

Further involvement of poultry: The virus then transferred to Oita prefecture, where mortality of bantams and a duck occurred in mid-February 2004, about 150 km from its initial occurrence. Towards the end of February, the virus caused mortality in chickens in Kyoto prefecture, about 350 - 450 km from its earlier presence. Few days later (3 March) the virus propelled a fourth and last outbreak in chickens, in the same town (Tamba) of Kyoto prefecture, where stamping out was completed on the 22 of March 2004 [7]. All commercial chicken farms and some hobby chicken farms in the movement control areas were subjected to sampling for serological examination and virus isolation, but neither AI antibody nor virus was detected [8].

Involvement of wild birds: Among the four outbreaks, the third one was considerably marked, in terms of both impact and duration, and was followed by lethal infection of nine crows within a 30-km radius of the afflicted farm in Kyoto, while the last virus-positive crow was found 1 month after the clean-up of that farm [7]. Faecal samples from wild birds and water samples from ponds and lakes in the movement control areas were also subjected to virus isolation attempts, but no AIV was isolated [8].

Contemporaneous and proximal occurrences of HPAIVs in neighboring countries: : South Korea reported an outbreak caused by HPAI H5N1 among ducks and chickens on the 10th of December 2003, which was the first official report of disease for this outbreak of H5N1 in Asia, and was the first recorded HPAI outbreak in South Korea's history. The outbreak happened in a chicken farm located in Eumsung district (Chungcheong-buk province, in the central part of the South Korea). Migrating birds have frequently been observed in a corn field next to the affected farm. A second event of HPAI was diagnosed in ducks at a farm located approximately 0.36 km from the first event, whereby only decreases in egg production and feed consumption rates, without clinical signs or mortality, were reported.

The duck farm likely was infected before the chicken farm, based on a serological investigation. There was no known movement of birds between the two farms, but it was found that the parent duck farm could have been the physical vector, since this farm also provided ducklings to a second farm that also became infected. HPAI H5N1 virus-infected poultry were identified on 17 additional farms by 20th of March 2004, the day when the last case was reported. Three magpies were found infected with the same virus near the two farms where H5N1 HPAI was confirmed, yet considering that magpies are a nonmigratory common bird in Korea and typically reside within 3.5 km of nest areas, it is likely that the magpies were infected from exposure to infected chickens, rather than vice versa [9].

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The representative isolated viruses - A/chicken/Korea/ES/03 and A/duck/Korea/ESD1/03 - phylogenetically contained HA and NA genomic segments (GSs) of the A/goose/Guangdong/1/96 (Gs/Gd) lineage, while the current H5N1 strains in Asia, including the Korean isolates, sharing a gene constellation similar to that of the Penfold Park virus, Hong Kong, isolated in late 2002 and containing some molecular markers that seem to have been fixed in the Gs/Gd lineage virus since 2001 [9].

Genomic characteristics of the viruses isolated in Japan: Viruses were isolated from all four outbreaks, and genetic analysis of representative viruses - such as A/chicken/Yamaguchi/7/2004 and A/chicken/Kyoto/3/2004 - showed clustering with a single genotype V virus, previously isolated in Shantou, in the Guandong province of mainland China [7]. The viruses introduced into both Japan and South Korea were, then, of the genotype V viruses, while those that became dominant in Vietnam, Thailand, Cambodia, Indonesia, and southern China were of the Z genotype virus, which first emerged in 2002, and has since become the dominant virus genotype in terrestrial poultry [10]. Alongside, it has been pointed out that the pertinent clade is clade 2.5, which includes the South Korean strain A/chicken/Korea/ES/2003, together with Japanese isolates such as A/chicken/Oita/8/2004, A/chicken/Kyoto/3/2004, A/chicken/Yamaguchi/7/2004, A/Crow/Kyoto/53/2004 and A/crow/Osaka/102/2004 [11].

Extraneous origin and spread in Japan: It has been suggested that the viruses isolated in the various mentioned prefectures in Japan were introduced into these prefectures on separate occasions from East Asia, by migratory birds [8]. Alternatively, the virus could have been transferred from one prefecture to another by wild waterfowl that were not detected, just as the initial ones remained undetected. At any rate, the 2003 - 2004 outbreaks are marked, outstandingly, in that only terrestrial - rather than aquatic - wild birds were found infected in Japan, alongside with the infected poultry. The involvement of infected crows is indeed noticeable, though. A large number of crows were observed near the chicken houses in Kyoto (after this outbreak started), indicating that the infected crows contracted the virus by direct contact with virus-contaminated material, probably through scavenging on dead chickens [7]. Since crow deaths were not observed prior to chicken deaths, and chickens did not have direct contact with crows, it is unlikely that chickens were infected from crows. Rather, it is plausible that the unaffected - and unrecognized, in that case, though likely to be waterfowl - migratory birds that introduced the pathogen into Japan, thereafter served as inland spreaders as well. Crows may be exceptionally sensitive, clinically - as is often the case with various terrestrial species - to these H5N1 viruses, although the extent of their infectedness, hence morbidity and mortality rates too, remain unknown. On the other hand, given that the last virus-positive crow was found 1 month after the clean-up of the farm in Kyoto, it is possible that the virus may have spread among crows at times sub-clinically, albeit but locally and temporarily.

In addition, important and unique findings were obtained regarding the role of blow flies as mechanical vectors of this HPAIV in Kyoto. H5N1 viruses were detected in 10-30% of the blow flies collected in the relevant areas, and in some cases viruses were isolated from the intestinal organs of the flies. The isolate A/blow fly/Kyoto/93/2004 was highly related to A/chicken/Kyoto/3/2004 and A/crow/Kyoto/53/2004, while viral viability lasted for 24 hours in crops and intestines of flies exposed experimentally to the virus, and for 48 hours in the flies feces and vomit matter [12]. Those findings are particularly significant, considering that all of the involved poultry farms had fowling nets in place to prevent entry of wild birds.

2007 - H5N1

Initial indication: On the 4th of January 2007, a dying mountain hawk-eagle was found in Kumamoto prefecture. HPAIV subtype H5N1 was isolated from both tracheal and cloacal swabs of the dead bird, designated A/mountain hawk eagle/1/Kumamoto/2007 [13].

Further involvement of wild birds: No further involvement of wild birds has been observed. Fecal samples from wild birds and water samples from ponds and lakes in the movement control areas were also subjected to virus isolation attempts, but no AIV was isolated allover [13].

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Involvement of poultry: : On the 13th of January 2007, illness (swollen faces) and increased mortality were observed in chickens in Miyazaki prefecture (Kyotake town). A second outbreak started on 22nd of January in the same prefecture (Hyuga city) in chickens, while in the same day another outbreak was observed in another prefecture, Okayama, Takahashi city, in chickens. The fourth and last outbreak started on the 30 of January in Shintomi town of Miyazaki prefecture. All commercial chicken farms and some hobby chicken farms in the movement control areas were subjected to sampling for serological examination and virus isolation, but neither AI antibody nor virus was detected.

Contemporaneous and proximal occurrences of HPAIVs in neighboring countries: HPAI H5N1 viruses were isolated in November 2006 in South Korea. Further outbreaks in ducks and chicken farms occurred across various districts of South Korea, such as Iksan, Asan, Ansung, Kimje and Cheon-An, until March 2007.

Genomic characteristics of the viruses isolated in Japan: TThe Japanese isolates clustered foremost with clade 2.2 H5N1 HPAIVs from South Korea and Mongolia, isolated in 2006, namely:

- A/duck/Korea/Asan/5/2006 (maximal homology)
- A/chicken/Korea/IS/2006
- A/whooper swan/Mongolia/2/2006
- A/common goldeneye/Mongolia/12/2006

The Japanese isolates grouped as well with the prototypic Chinese virus of 2005, A/black headed goose/Qinghai/1/2005 [11].

Extraneous origin and spread in Japan: This HPAIV occurrence lasted in Japan for about one month. The outbreaks in South Korea started about one month before the initial indication (the infected mountain hawk-eagle) surfaced in Japan, an interval during which transfer of the virus from South Korea to Kumamoto, plausibly through subclinically infected waterfowl, could readily take place. Subsequent virus transfer from Kumamoto to Miyazaki, south-eastwards, could similarly proceed. This spatio-temporal course is as well supported by the phylogenetic findings described above.

The mountain hawk-eagle contracted the virus apparently through preying on an infected bird. Since mountain hawk-eagles are resident in Japan, with habitat range not exceeding 25 km, it is improbable that this species accounted for either the introduction of the virus to Japan, or for the virus transfer from Kumamoto to Miyazaki (75 km) [13]. In similarity to the 2003 - 2004 H5N1 occurrences in Japan, during the 2007 occurrences no involvement of wild aquatic birds has been observed, yet this cannot be regarded as an indication that such involvement did not play a role in actuality, and drove both virus introduction into and spread in Japan.

2008 - H5N1

Initial indication: Four dead whopper swans were found in lake Towada, Aomori prefecture, on the 21st of April 2008. The causative agent was found to be HPAIV H5N1, generating severe congestive edema in the lungs of the dead swans [14,15].

Further involvement of wild birds: More dead or moribund whooper swans were found during April and May 2008 in the nearby Akita prefecture, as well as in Hokkaido, until the beginning of May. The H5N1 HPAIV causing the same pathology mentioned above, was isolated repeatedly [14,15].

Involvement of poultry: Poultry has not been involved during 2008.

Contemporaneous and proximal occurrences of HPAIVs in neighboring countries: Outbreaks in chickens occurred in April 2008 in Primorsky, Russia (south-eastern corner of Siberia, opposite to Hokkaido), upon spring migration of waterfowl, which were found infected too [16]. Thereby, typical isolates, such as A/chicken/Primorje/1/08 and A/Anas crecca/Primorje/8/08 were affiliated with the 2.3.2. clade, a clade then appearing for the very first time in Russia. Alongside, it has been accentuated that about 25% of the wild birds, which include mainly common (Eurasian) teals (*Anas crecca*), mallard ducks (*Anas platyrhynchos*), and great-crested grebes (*Podiceps cristatus*), were involved in viral circulation in the watery area complex of the Suifun-Khankai plain of Primorsky region [17].

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Genomic characteristics of the viruses isolated in Japan: Typical isolates, such as A/whooper swan/Aomori/1/2008, A/whooper swan/Akita/1/2008 and A/whooper swan/Hokkaido/1/2008, were found affiliated with clade 2.3.2, together with A/chicken/Primorje/1/2008 (maximal homology), and earlier strains from ducks, isolated in China and Vietnam, like A/duck/Guangxi/345/2005 and A/duck/Vietnam/568/2005 [11]. Thus, the Japanese strains were nearly identical to chicken viruses isolated in Primorsky, Russia, in April 2008, and closely related to viruses isolated from dead wild birds in Hong Kong in 2007-2008, while their HA genomic segments clustered in clade 2.3.2. On the other hand, the NA and the internal genomic segments were closely related to those of clade 2.3.4 viruses (genotype V), whose NP gene originated from an HA clade 2.3.2 virus. It can therefore be concluded that the H5N1 viruses isolated in Japan, Russia and Hong Kong were derived from a common ancestor virus belonging to genotype V, which was generated through genetic reassortment events between viruses of HA clades 2.3.2 and 2.3.4 [18]. HPAIVs isolated in Korea and China were also found - in regard to the HA - phylogenetically related to the Japanese viruses [15].

Extraneous origin and spread in Japan: TThe origin of the swan virus in Japan is in this case somewhat obscure, although it is fairly clear that the virus was conveyed from north Japan northwards, to the quite adjacent Primorsky region of Russia, very soon after it infected swans in Japan. The common vector host could be long-distance south-to-north sub-clinically infected migratory waterfowl, possibly originating from South-East Asia and engaging the Australian-East Asian flyway, to Siberia. This is compatible with the phylogenetic affinity found between the Japanese strains and homologous strains from Vietnam and Hong Kong, as mentioned. A South-to-North axis from Vietnam to Hong Kong actually heads further strait to Japan.

Notably, in Japan about 35,000-38,000 whooper swans spend every winter primarily in Hokkaido, Tohoku (the northeastern portion of Honshu), and Hokuriku (northwestern Honshu). In the Lake Towada area, Aomori, about 300 whooper swans arrive, beginning in late October, and leave between late March and late April. In late March, summer birds begin to arrive. According to results of satellite tracking of 8 swans, as well as of banding studies since 1961, whooper swans that winter in Japan migrate from the northern end of Honshu Island to eastern Hokkaido, thereafter, via Sakhalin, reaching eastern Siberia, where they breed [15].

In light of the migratory route mentioned above, the whooper swans found dead at Lake Towada were most likely recently infected with HPAIV (H5N1) in Japan. It is nearly improbable that the swans were infected before they flew to Japan in autumn and maintained a LP form of the virus within the flock, which then became HP. Although the susceptibility of a certain species of birds to HPAI H5N1 viruses may appreciably vary, depending on the virus strain, whooper swans, as well as mute swans, have been considered to be clinically susceptible species to HPAIV (H5N1), as they showed a fulminant course of disease on various occasions of natural infection. Experimentally, swans - including whooper swan - were found extremely affected by HPAI H5N1 challenge, in comparison to geese [19], (let alone in comparison to ducks). The possibility that the swans were infected with HPAIV by poultry is most unlikely, since HPAI in poultry has not been observed in Japan throughout 2008.

A plausible source of the 2008 infection could be that other wild birds conveyed the virus from outside Japan. Although it is not known whether any birds wintering on the continent migrate northwards through Japan, passage visitor birds such as wader birds migrate from south to north through Japan in spring via the Australian-East Asian flyway. Besides, summer birds such as egrets, swallows, songbirds, and some raptors, come to Japan from the south in spring for breeding. Alongside, the phylogenetic analysis demonstrated that the virus that killed the swans in Japan in 2008 is genetically distinguishable from the strains that occurred earlier in Japan, thus ruling out a possibility of resurgence of previously introduced HPAIV in Japan [14].

The appearance of HPAI H5N1 in Hokkaido in 2008 is notable, considering that from 1996 to 2009, every autumn, fecal samples of migratory water birds were systematically collected in Hokkaido and Mongolia, yielding a total of 634 viruses, including 17 LPAI H5 viruses, of which only one, isolated in 2009 from a mallard - A/mallard/Hokkaido/24/09 - was H5N1 [20].

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2010-2011 - H5N1

Initial indication: Two HPAI H5N1 isolates were obtained on the 14th of October 2010 in Hokkaido, from fecal samples of apparently healthy wild ducks, upon their autumn migration from their Siberian breeding grounds. The samples were collected at Lake Onuma in Wakkanai, the northernmost stopover site of the birds in Japan [21]. The isolated viruses have been genomically affiliated with Group C (all in all, as characterized below, there were three groups, namely, A, B and C).

Further involvement of wild birds: Thereafter, all occurrences in Hokkaido, from January 2011 onwards, were only from the eastern Kushiro area, 350 km south-east of Lake Ohnuma. The isolated viruses, together with wild waterfowl isolates from Fukushima, have been affiliated with Group A. Mostly consecutively, until March 2011, many additional H5N1 isolates were obtained across Japan from a wide variety of apparently healthy wild aquatic birds, both migratory and resident, plus several dead raptors. All in all, 63 isolates were obtained from wild birds in 17 prefectures, predominantly in the region of Kyusyu. The infected wild birds comprised 17 species, consisting of 14 water birds and 3 raptures [21].

The infected wild birds included the following species [22]:

- Whooper swan (*Cygnus cygnus*)
- Black swan (*Cygnus atratus*)
- Tundra swan (*Cygnus columbianus*)
- Mute swan (*Cygnus olor*)
- Tufted duck (*Aythya fuligula*)
- Greater scaup (*Aythya marila*)
- Common pochard (*Aythya ferina*)
- Northern pintail (*Anas acuta*)
- Mandarin duck (*Aix galericulata*)
- Little grebe (*Tachybaptus ruficollis*)
- Great crested grebe (*Podiceps cristatus*)
- Hooded crane (*Grus monacha*)
- Grey heron (*Ardea cinerea*)
- Black headed gull (*Chroicocephalus ridibundus*)
- Peregrine falcon (*Falco peregrinus*) (many isolates)
- Northern goshawk (*Accipiter gentilis*)
- Ural owl (*Strix uralensis*)

Among the various HPAI occurrence events in Japan, this event is marked by the widest diversity of wild birds found infected, and accentuates their potential in virus circulation. Except for the two initial wild duck isolates from Hokkaido, the other wild bird isolates from Hokkaido and Fukushima (north Japan) solely comprised Group A (classified in that case according to their HA phylogenetic profile), while Group B viruses occurred across a small quadrate territory bounded by Tottori-Toyama-Aichi-Tokushima, predominantly within wild birds (and only one isolated outbreak in chickens, in Aichi). Occurring only in two northern locations - Ohnuma (Hokkaido) and Aomori - Group C viruses were isolated mostly from Tochigi southwards [22].

Involvement of poultry: During November 2010 to March 2011, multiple episodes of H5N1 outbreaks in chicken farms occurred across Japan. All in all, there were outbreaks in 24 chicken farms in 9 prefectures [21], predominantly in the region of Kyusyu, yet only from Chiba southwards, while mainly the Group C viruses (mentioned below), were involved; Group B viruses generated outbreaks in chickens but sporadically, in Aichi and Tottori, whereas Group A viruses caused no outbreaks in chickens [22].

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Contemporaneous and proximal occurrences of HPAIVs in neighboring countries: On December 7, 2010, a H5N1 HPAIV was isolated from a healthy mallard captured at the Mankyung River in South Korea. Phylogenetic analysis showed that this virus was classified into clade 2.3.2, and closely related to H5N1 viruses isolated from wild birds in Mongolia, Russia and China in 2009 and 2010 [23]. Out of 728 fresh fecal samples collected while surveying Korean wild bird habitats during the winter of 2010-2011, 14 HPAI H5N1 viruses were identified. The isolates phylogenetically clustered with other recently isolated clade 2.3.2 HPAI H5N1 viruses from wild birds in Mongolia. Twelve of the 14 HPAI-positive samples were typed as Mandarin Duck (*Aix galericulata*). Notably, no dead or diseased mandarin ducks were found across the sampling sites [24]. Outbreaks in chicken farms in South Korea were first observed in December 2010 and continued during the winter into 2011.

Genomic characteristics of the viruses isolated in Japan: Genomic homology was foremost revealed towards Mongolian and Korean isolates, basically within the 2.3.2 clade. The H5N1 HPAIVs initially isolated in Hokkaido in October 2010 from wild ducks were almost identical to the H5N1 HPAIVs isolated from dead whooper swans in May 2009 and May 2010 in Mongolia, and viruses most closely related to those Group C Hokkaido strains were subsequently isolated until December 2010 from domestic and wild birds at multiple distinct sites across Japan, where the migratory flyways of the waterfowl overlap [25].

Another strain from a whooper swan in Hokkaido in 2011 - A/Ws/Hokkaido/4/11 (as well as 2009-2010 Mongolian-like clade 2.3.2 isolates) - was found to have sequence homology as high as 99.8% with HPAI H5N1 viruses - A/Md/Korea/W401/11 and A/Md/Korea/W404/11 - isolated in Korea during 2011 from fecal samples of mallard ducks [26]. Similarly, a virus isolated in Japan from a wild duck (in Kochi Prefecture, south-western Japan) - A/mandarin duck/Kochi/3901C005/2011(H5N1) - had the highest nucleotide identity of the HA gene with A/Md/Korea/W401/2011 (H5N1) and A/mandarin duck/Korea/K10-515/2011 (H5N1), isolated in Korea in the 2010-2011 winter season [21]. Some Japanese strains from Yamaguchi prefecture too clustered with several Korean viruses isolated in south-middle Korea [22].

In the latter study, the 63 viruses isolated in Japan during 2010-2011 were phylogenetically analyzed in reference to their HA gene and thereby divided into the three groups A, B and C, while Group C differentiating into 6 subgroups. In similarity, Sakoda., *et al*. [21] profiled the HA of about half of the isolates, and found that in group B, all viruses were isolated only from western areas of Japan (Aichi, Kyoto, Hyogo, Tokushima and Shimane), while in group C, viruses were isolated from the whole of the country (Hokkaido, Aomori, Tochigi, Aichi, Mie, Tottori, Yamaguchi, Kochi, Oita, Nagasaki, Miyazaki and Kagoshima). The PB2 and PB1, NP, NA and M genomic segments of the isolates were as well divided into three genetic groups, corresponding to the classification of the HA genes, except for a few unaffiliated isolates. Also, the PA and NS genomic segments were not classified completely into groups A, B or C [21]. The latter study observed, however, that the three classified A, B and C group viruses were transferred to Japan by migratory waterbirds through at least three different routes from their northern summer Siberian territories.

At any rate, all genomic segments, except the PA, were found closely related to Japanese wild bird isolates of 2008, and belonged to clade 2.3.2.1, which contains viruses from China, Russia, Mongolia and Korea [27]. Direct ancestors of the PA gene segment of all the Japanese 2010-2011 isolates are found in wild bird strains of several antigenic subtypes other than H5N1, isolated between 2007 and 2009. The PA genomic segment of these wild bird isolates shares a common ancestor with H5N1 HPAIVs belonging to clades 2.5, 7 and 9, indicating that wild birds were involved in the emergence of the 2010 reassortant 2.3.2.1 viruses isolated in Japan [27].

Extraneous origin and spread in Japan: : It appears that the proximal source of the initial (October 14th 2010) H5N1 HPAIV in Japan was infected, yet unaffected, wild waterfowl that migrated from East Siberia directly - via Sakhalin - to Lake Onuma in Wakkanai, Hokkaido, the northernmost stopover site of the birds in Japan. Still, it is likely that three migration paths were independently involved in the conveyance of HPAIVs to Japan during October-November 2010: an early one in the first half of October, from East Siberia to Hokkaido; a second one in the second half of October, from central Siberia and Mongolia, via South Korea, to Yamaguchi prefecture and the Kyusyu region; and a third one in the first half of November, from Western Siberia to middle and/or south Japan, once again via South Korea, or

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via China. Notably, this pattern may well resemble the one that has clearly been evidenced in regard to the phylogeographic profile of the 2014 H5N8 HPAIV in Japan (as elaborated on below).

An extended retrospective observation based on the spatiotemporal and phylogenetic profiles of the viruses isolated in Japan suggests that the they experienced a north-south round trip in eastern Eurasia during 2009 - 2010, meaning: wild waterfowl contracted the virus from poultry in China, migrated to Siberia via Mongolia, and later on, migratory waterfowl such as pintail ducks conveyed the virus from Siberia to Japan. Also, the initial Hokkaido strain was found to be appreciably less pathogenic in experimentally infected ducks than the homologous 2009 Mongolian isolate, implying, importantly, of attenuation that possibly took place while the virus perpetuated within wild waterfowl [25].

It has further been postulated that the perpetuated viruses circulating within different populations of migratory birds across their breeding grounds in Siberia during summer 2010 were indeed subsequently conveyed to Japan through at least three different migratory waterfowl routes, via China, Korea and Russia; and then ongoing virus spread in Japan occurred within wild birds at the lakes occupied by them through water-borne transmission and predation of infected carcasses [21].

Noticeable are all the viruses in subgroups C-1 and C-6, in that they were isolated only in the Kyusyu region of Japan, indicating that these viruses were maintained in local bird populations during the outbreak periods. Moreover, the viruses in these two subgroups were isolated from poultry in addition to resident birds, suggesting that direct or indirect viral transmission among them occurred in each prefecture. All the isolates from hooded cranes at the Izumi migration grounds in Kagoshima prefecture were classified into another distinct branch - Subgroup C-5, while the fact that the crane viruses were isolated in December 2010 and February 2011 implies that they have been circulated within the related crane population for 2 months [22].

On the whole, different viruses persisted in Japan at least from 14 October 2010 to 6 March 2011 (144 days), a period nearly strictly dovetailing the presence of wintering migratory birds in Japan. It plainly reflects, correspondingly, an entire course of HPAIV conveyance to, spread across, partial perpetuation in, and vanishing from Japan. Resident avifauna and poultry did not have the capacity to sustain lasting virus circulation (beyond the wintering season), an incompetence which is extremely desirable, yet largely - and apparently vitally - assisted by systematic culling and rigorous control procedures.

The postulation that HPAIVs invaded Japan during autumn 2010 via three different independent migration routes originating from three Siberian and Mongolian reproduction focuses is indeed particularly meaningful. Significantly, it overlaps, in principle, with the pattern later on observed in regard to the remarkable occurrences of H5N8 HPAIVs in Japan, which took place during 2014 - 2015 (as detailed and compared with below), and underscores Eastern, Central (together with north Mongolia), and Western Siberia as three prime ecogenetic melting pots of IAVs.

2014-2015 - H5N8

Initial indication: An isolated outbreak occurred in chickens in Kumamoto Prefecture, Kuma-Gun district, in April 2014. A HPAIV of the subtype H5N8 was isolated on the 13th of April 2014, for the first time ever in Japan. The outbreak lasted for three days, until total culling. After this single outbreak, no HPAIVs were detected or isolated in Japan until the end of October 2014.

Further involvement of poultry: Four outbreaks in chicken farms occurred during December 2014-January 2015, as follows [6,28].

• In Miyazaki-shi, Takaoka-cho, Miyazaki - 15/12/2014;
• In Yamaguchi - 30/12/2014;
• In Kasaoka-shi, Okayama - 15/01/2015;
• In Arita-cho, Saga - 17/01/2015.

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A list of the viruses isolated throughout 2014 - 2015 is presented below with their genomic characteristics.

Involvement of wild birds: On the 3rd of November 2014, in Yasugi-shi, Shimane, two fecal samples from apparently healthy tundra swans were collected and found to contain H5N8 HPAIVs. This was the first time a HPAI H5N8 virus has been ever isolated from a wild bird in Japan [29].

Fecal samples from migratory ducks revealed the presence of HPAI H5N8 viruses in Chosei-gun, Nagara-machi, Chiba and in Tottori-shi, Tottori, on the 18th of November. On 23 November 2014, an incapacitated white-naped crane was sampled at the Izumi plain (a vast territory occupied by wintering cranes and waterfowl in Kagoshima, south Kyushu) and found to be infected with HPAI H5N8. Due to the presence of the latter virus, active surveillance of wild birds within 10 km radius was conducted and yielded eight H5N8 HPAIVs from six incapacitated or dead cranes (both hooded and white-naped), two dead mallards, and a water sample (1st of December) collected from the cranes wintering grounds, until the 13th of February 2015 [6,29]. One isolate from a dead Mandarin duck (*Aix galericulata*) was obtained in Kani-shi, Gifu, on the 12th of December [6]. A list of the viruses isolated throughout 2014-2015 is presented below, with their genomic characteristics.

Contemporaneous and proximal occurrences of HPAIVs in neighboring countries: HPAI H5N8 viruses were isolated in China from apparently healthy mallard - A/mallard duck/Shanghai/SH-9/2013 (H5N8) - in November 2013, as well as from domestic ducks in November 2013 and January 2014, namely the viruses A/duck/Beijing/FS01/2013 and A/duck/Beijing/FS01/2014, respectively.

Further, on January 16, 2014, mild clinical signs of HPAI, such as decreased egg production (60%) and slightly increased mortality rates, were detected in a duck farm near the Donglim Lake Reservoir in Jeonbuk Province, South Korea; and on January 17, a farmer (5 km from the Donglim Lake Reservoir) also reported clinical signs of HPAI in another duck farm. Concomitantly, about 100 carcasses of Baikal teals were found in the Donglim Lake Reservoir, which is known to be occupied every winter by about 200,000 wintering Baikal teals [30].

In addition, 37 HPAI H5N8 viruses isolated from wild birds and poultry farms in South Korea until May 8 2014, showed that all of the isolates belonged to clade 2.3.4.6 of the HA gene, but comprised two distinct groups. During the initial stage of the outbreak, identical isolates from each group were found in wild birds and poultry farms near Donglim Lake Reservoir, which is a resting site for cranes plus various migratory birds too, thereby indicating that two genotypes of H5N8 HPAIVs were introduced into the lake at the same time [31].

Phylogenetically, the Chinese HPAI H5N8 virus isolated from an apparently healthy mallard in November 2013 - A/mallard duck/Shanghai/SH-9/2013 (H5N8) - was probably homologous to the pertinent virus initially transmitted from China to South Korea, considering that Shanghai is a desirable site to monitor influenza virus in China, as it is located on the East Asia migratory bird flyway, which spans portions of China, South Korea and Japan. Among the three South Korean strains Gochang1, Buan2 and Donglim3, Gochang1 falls into the same clade with A/mallard duck/Shanghai/SH-9/2013, while nucleotide identities are of 98-99%. The origins here were also duck viruses, alongside with an environmental isolate: A/duck/Jiangsu/k1203/2010 (H5N8) (mentioned above), A/duck/Hunan/S11643/2013 (H4N9), and an H11N9 environmental isolate collected in Jiangxi province too. The 2010 H5N8 virus was already HP [28,32].

Genomic characteristics of the viruses isolated in Japan: The 2014-2015 H5N8 incursion event into Japan provides an outstanding opportunity to intactly and meticulously investigate the ecophylogeographic dynamics of a vast system. A comprehensive analysis of the HA gene, rooted within a basal group containing two HPAI H5N8 viruses isolated from ducks in China in November 2013 and January 2014 (A/duck/Beijing/FS01/2013 and A/duck/Beijing/FS01/2014), includes phylogenetic and spatiotemporal data relating to a wide range of Japanese and other HPAI H5N8 viruses [28]. In tables 1, 2, 3 and 4, these data are systematically compiled into four sections - Prototype group; Genotype Sub-group G; Genotype Sub-group B; and Genotype Sub-group R - within a chronological pattern that enables to trace and follow the related evolutionary and geographic courses.

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Date of isolation	Country	Strain isolated
Initial virus isolated in Japan:		
13 04 2014	Japan	A/chicken/Kumamoto/1-7/2014
Earliest viruses isolated from wild and domestic birds outside of Japan (later than the two mentioned Chinese isolates used as basal group for this Phylogenetic analysis):		
19 01 2014	South Korea	A/bean goose/Korea/H40/2014
28 01 2014	South Korea	A/breeder chicken/Korea/H250/2014
Latest viruses isolated from wild and domestic birds outside of Japan:		
29 01 2014	South Korea	A/white-fronted goose/Korea/H231/2014
24 11 2014	South Korea	A/Korean native chicken/Korea/H1903/2014

Table 1: HA genotypic homologies and related spatiotemporal features of the HPAIV H5N8 Prototype Group that initially appeared in Japan in 2014.

Date of isolation	Country	Strain isolated
Viruses having ancestral linkage:		
17 01 2014	South Korea	A/broiler duck/Korea/Buan2/2014
06 02 2014	South Korea	A/tundra swan/Korea/H411/2014
08 02 2014	South Korea	A/common teal/Korea/H455-30/2014
02 03 2014	South Korea	A/breeder chicken/Korea/H818/2014
Viruses isolated in Japan:		
03 11 2014	Japan	A/tundra swan/Shimane/41/2014
18 11 2014	Japan	A/duck/Chiba/26-372-48/2014
18 11 2014	Japan	A/duck/Chiba/26-372-61/2014
01 12 2014	Japan	A/environment/Kagoshima/KU-ngr-H/2014
Earliest viruses isolated from wild and domestic birds outside of Japan:		
25 09 2014	Russia	A/wigeon/Sakha/1/2014
04 11 2014	Germany	A/turkey/Germany-MV/R2472/2014
24 11 2014	Netherlands	A/Eurasian_wigeon/Netherlands/emc-1/2014
01 12 2014	South Korea	A/mallard/Korea/H1924-6/2014
15 12 2014	Italy	A/turkey/Italy/14VIR7898-10/2014

Table 2: HA genotypic homologies and related spatiotemporal features of the HPAIV H5N8 Genotype Sub-group G.

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Date of isolation	Country	Strain isolated
Viruses having ancestral linkage:		
22 1 2014	South Korea	A/Baikal teal/Korea/H68/2014
17 3 2014	South Korea	A/breeder chicken/Korea/H1068/2014
Viruses isolated in Japan:		
15 12 2014	Japan	A/chicken/Miyazaki/7/2014
16 12 2014	Japan	A/mandarin_duck/Gifu/2112D001/2014
17 12 2014	Japan	A/crane/Kagoshima/KU21/2014
29 12 2014	Japan	A/chicken/Yamaguchi/6/2014
03 01 2015	Japan	A/crane/Kagoshima/KU53/2015
14 01 2015	Japan	A/mallard duck/Kagoshima/KU70/2015
15 01 2015	Japan	A/chicken/Okayama/1-2/2015
17 01 2015	Japan	A/chicken/Saga/1-1/2015
13 01 2015	Japan	A/mallard duck/Kagoshima/KU116/2015
Earliest and latest viruses isolated from wild birds outside of Japan:		
16 12 2014	South Korea	A/spot-billed duck/Korea/H1981/2014
19 12 2014	South Korea	A/mallard/Korea/H2003/2014

Table 3: HA genotypic homologies and related spatiotemporal features of the HPAIV H5N8 Genotype Sub-group B.

Date of isolation	Country	Strain isolated
Viruses having ancestral linkage:		
23 01 2014	South Korea	A/Baikal teal/Korea/H96/2014
08 05 2014	South Korea	A/gadwall/Korea/H1351/2014
Viruses isolated in Japan:		
18 11 2014	Japan	A/tundra swan/Tottori/C6nk/2014
23 11 2014	Japan	A/crane/Kagoshima/KU1/2014
07 12 2014	Japan	A/crane/Kagoshima/KU13/2014
Earliest and latest viruses isolated from wild and domestic birds outside of Japan:		
02 12 2014	Canada	A/turkey/Canada/FAV10/2014(H5N2)
08 12 2014	USA	A/pintail/Washington/40964/2014(H5N2)
08 12 2014	USA	A/gyrfalcon/Washington/41088-6/2014(H5N8)
16 12 2014	USA	A/chicken/Oregon/41613-2/2014(H5N8)
11 01 2015	Taiwan	A/goose/Taiwan/a015/2015 (H5N8)
13 01 2015	Taiwan	A/duck/Taiwan/a043/2015 (H5N2)
16 01 2015	Taiwan	A/chicken/Taiwan/a174/2015 (H5N3)

Table 4: HA genotypic homologies and related spatiotemporal features of the HPAIV H5N8 Genotype Sub-group B.

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Table 5 systematically summarizes the features of the earliest and latest HPAI H5N8 genotypes of viruses isolated in Japan during 2014-2015. Remarkably, the three genotypes co-circulated in late 2014 in Kagoshima (Izumi), and across South Japan in general, compatibly with further findings obtained in Japan [29]. The analysis [28] underscores the presence of South Korean strains - in January 2014, already - that show ancestral linkage to each of the three different Japanese genotypes, concretely, as well as to the Kumamoto prototype strain. Elsewhere, is has been contended that the Japanese and European H5N8 sequences all form a single cluster closely related to, but distinct from those found in the South Korean outbreak [33]. Additional findings, referring specifically to the April 2014 prototype A/chicken/Kumamoto/1-7/2014, pointed at its affiliation to the HA clade 2.3.4.4, while all eight genomic segments exhibiting particularly high sequence similarity to those of the H5N8 subtype HPAIVs A/broiler duck/Korea/Buan2/2014 and A/Baikal teal/Korea/Donglim3/2014, which were isolated in South Korea in January 2014 [34].

Entity	Host category	Date	Host	Place
Prototype	Domestic Bird	13 04 2014	Chicken	Kumamoto
		16 04 2014	Chicken	Kumamoto
	Wild Bird	None		
Genotype G	Domestic Bird	None		
	Wild Bird	03 11 2014	Tundra Swan	Shimane
		01 12 2014	Environment	Kagoshima
Genotype B	Domestic Bird	None		
	Wild Bird	18 11 2014	Tundra Swan	Tottori
		07 12 2014	Crane	Kagoshima
Genotype R	Domestic Bird	15 12 2014	Chicken	Miyazaki
		17 01 2015	Chicken	Saga
	Wild Bird	16 12 2014	Mandarin Duck	Gifu
		13 01 2015	Mallard Duck	Kagoshima

***Table 5:** Features of earliest and latest HPAI H5N8 genotypes of viruses isolated in Japan during 2014 - 2015.*

Extraneous origin and spread in Japan: The isolated three days-outbreak in a chicken farm in Kumamoto, Japan, in mid-April 2014, with no further virus isolation until the end of October, probably means that there were two independent introductions of H5N8 viruses into Japan, in April 2014 and in November 2014. Sakoda indicated [35], that the April 2014 virus was transferred from the Chinese area of Shandong to South Korea, and therefrom to Kumamoto, which is compatible with the analysed phylogenetic affinities of the prototype Kumamoto virus. Within such scenario, could have been involved the early, above mentioned Chinese HPAIV A/mallard/Shanghai/SH-9/2013(H5N8).

Alongside, though, the initial genesis of the Kumamoto strain has been traced back to 2009, pointing at the South Korean H5N8 duck isolate - HPAI, already - A/mallard duck/Korea/W452/2014(H5N8), as the direct precursor virus, preceded by five different reassortant viruses isolated consecutively from merely ducks in China since 2009 - of which A/duck/Jiangxi/28/2009 (H11N9) was the primal precursor virus - and leading, apparently through the Chinese HPAI duck strains:

- A/duck/Jiangsu/k1203/2010 (H5N8),
- A/duck/Zhejiang/W24/2013 (H5N8),
- A/mallard/Shanghai/SH-9/2013 (H5N8)

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To the emergence of three South Korean HPAI H5N8 viruses with full homology to the Kumamoto strain [36], namely:

- A/Baikal teal/Korea/Donglim3/2014(H5N8),
- A/duck/Korea/Buan2/2014(H5N8), and
- A/broiler duck/Korea/Gochang1/2014 (H5N8).

Plausibly, the H5N8 HPAIV reached Japan for the first time through a mallard moving from South Korea, and carrying a virus equivalent to A/mallard duck/Korea/W452/2014(H5N8). This postulation is consistent with findings obtained for experimental infection of ducks with the Kumamoto strain, demonstrating that the infection was productive, yet asymptomatic, in all challenge doses examined [37].

The phylogenetic affinities of the various H5N8 strains that were isolated in Japan from November 2014 to February 2015 (all in all 103 days), are much more complex then the H5N8 Kumamoto strain. Far back, all the mentioned HPAI H5N8 viruses shared an early common ancestor, in that their HA and PB2 genomic segments were derived from the A/Goose/Guangdong/1/96 (H5N1) lineage [38]. More specifically, the genotypic sub-groups B, R and G have also equivalent designations, A2, A3, and A1, respectively, while A2 allegedly originating in Eastern Siberia, A3 in Central Siberia, and A1 in Western Siberia, all during spring-summer 2014 [39]. This implies of a vast Siberian area that had been seeded by infected waterfowl coming from Mongolia, China and Korea in spring 2014 with the prototypic virus, and during the next six months gave rise to three different genotypes across eastern, central and western Siberia, correspondingly, all thereafter conveyed to South Korea and Japan by migratory waterfowl, in fall 2014.

For two genotypes (sub-group G, also designated A1, and sub-group B, also designated A2), the related viruses were apparently introduced into Japan by subclinically infected Tundra swans, which was possibly the case for the third genotype (sub-group R, also designated A3) too. It is of note that the third genotype was isolated also from a waterfowl, Mandarin duck, which is a native all-year resident wild duck species in Japan. Mallard ducks, one of the most important hosts of IAV, were involved as well. Significantly, all the three genotypes were isolated in Kagoshima, which contains an outstanding wintering site for cranes in Japan (Izumi), and is likely frequented by water birds coming from most parts of Siberia. Japan, specifically Kagoshima, is unique within that context, although the three genotypes were isolated in South Korea as well, in general. The fact that the A1 genotype, which emerged in West Siberia, was isolated from a strongly migratory duck (wigeon) also in Sakha, East Siberia, is remarkable, underscoring the tremendous mobility of AIVs along a West-East Siberian axis, which, in conjunction with the obvious North-South migration axes, are of particular implications for continental East Asia, as well as for Japan. Consequently, the ecophylogeographic amplitude affecting continental East Asia and Japan is by far extended, to Europe and to America. Such a broad perspective has been supported by additional findings [38]. Interestingly, yet, the presence of South Korean strains - in January 2014, already - that show ancestral linkage to each of the three different genotypic groups detected in Izumi cranes wintering compound, Japan, has been pointed at [28].

Considering that wild ducks also overwinter in the Izumi wintering ground of the cranes, it has been assessed that this watery territory may serve for virus transmission, and this has been evidenced, typically, with regard to LPAI viruses [40]. A part of the Izumi plain, the Izumi crane wintering grounds, which cover a 245ha wet paddy field area in the northwest of Kagoshima Prefecture, is known for the roughly 10,000 cranes which pass the winter therein every year, from mid-October to March. The wet paddy areas are artificially created to provide roosting sites for the cranes every winter. Collectively, then, while it is clear that the Chinese duck viruses constituted the original melting pot underlying the genesis of the HPAI H5N8 viruses at large, the Donglim Lake Reservoir in Korea and the Izumi watery plain in Japan played a major role in the ecoepidemiology of those viruses, parallel to Siberia.

2016-2017 - H5N6

This HPAI subtype was detected for the very first time ever in Japan on 6 November 2016, persisted until 24 March 2017, and reappeared on 7 November 2017. Comprising fresh invasions, it is reviewed here but briefly.

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During the initial phase of virus introduction, along November, the following isolates were obtained in various detached locations across Japan from wild birds and environmental water, chronologically:

- A/Northern Pintail/Tottori/b37/2016(H5N6) – 2016-11-06
- A/duck/Hyogo/1/2016(H5N6) – 2016-11-14
- A/environment/Kagoshima/KU-ngr-I/2016(H5N6) – 2016-11-14
- A/teal/Tottori/1/2016(H5N6) – 2016-11-15
- A/black swan/Akita/1/2016(H5N6) – 2016-11-19
- A/crane/Kagoshima/KU-4/2016(H5N6) – 2016-11-19
- A/white-fronted goose/Miyagi/1/2016(H5N6) – 2016-11-21
- A/whooper swan/Iwate/1/2016(H5N6) – 2016-11-23

Additional isolates from wild birds were obtained later from various swans species, foremost, as well as from aquatic birds such as white fronted goose, coot, plus birds of prey, namely peregrine falcon, grey-faced buzzard, and owl [41].

Initial outbreaks occurred, untypically, in a zoo and a Muscovy duck farm. Two black swans at Higashiyama Zoo and Botanical Gardens in Nagoya (central Japan) were lethally infected in mid-November in an open-air pond near the main gate, where wild birds can fly in and out. About ten days later, an H5N6 HPAIV appeared in a Muscovy duck farm in Aomori (north Japan), and four days later brought about the same occurrence in a second Muscovy duck farm located 350 meters from the first one, and owned by the same company. At about the same time, outbreaks occurred at two layer chicken farms in Niigata prefecture - yet 143 km from each other - on November 29 and 30, 2016, and later on, until March 2017, at ten additional chicken farms across further seven prefectures throughout Japan at large. Overall, about 1.6 million chickens and 23,000 Muscovy ducks died or were euthanized during that epizootic, while the index isolates have been A/Muscovy duck/Aomori/1-3T/2016 and A/chicken/Niigata/1-1T/2016 [42]. According to this source, two NS clusters and a PA cluster in Japanese H5N6 HPAIVs originated from Chinese H5N6 HPAIVs, whereas three ordinary AIV-derived PA clusters (from wild birds in China, Mongolia, and Europe) were evident.

Elsewhere, it has been found that a phylogenetic tree analysis of the HA gene revealed that the H5N6 isolates are classified into the genetic clade 2.3.4.4c and cluster with the recent H5N6 HPAI isolates from wild and domestic birds and humans in China, in addition to a wild duck isolate from South Korea, A/Mandarin duck/Korea/K16-187-3/2016 (H5N6). The NA genes of the isolates also form a single cluster together with the H5N6 HPAIV isolates from China. In addition, the remaining 6 genomic segments were genetically close to the recent H5N6 HPAIV isolates from China, except for the PB1 genes, which are most closely related to the counterpart of A/duck/Guangdong/S4040/2011(H4N2), isolated from a domestic duck at a live bird market in China [43].

The HPAI H5N6 virus came into being apparently in 2013 in China, in the form of the prototype duck strain A/duck/Jiangsu/WX156/2013(H5N6), and considerably evolved since. It may be concluded that the virus was introduced - once, or more likely, repeatedly - from China, where it is endemic. Subclinically infected Anatide birds migrating from China and/or South Korea to Japan probably introduced the virus, apparently in a nearly simultaneous multi-route pattern. While a similar introduction machinery into Japan has been evidenced already, the 2016-2017 event is particularly marked by the initial outbreaks in muscovy duck farms, and by the H5N6 reappearance in November 2017. The affinity to farmed muscovy ducks may be incidental, or can be linked to the fact that muscovy ducks were involved, quite exceptionally, in the evolving of the HPAIV H5N6 in Vietnam, through the strain A/Muscovy duck/Vietnam/LBM631/2014(H5N1) [71]. Remarkable as well is the fact that six and a half months after the H5N6 virus apparently disappeared from Japan, it reappeared in Shimane Prefecture and was isolated from mute swan.

Infections of Mammals with HPAIVs in Japan

In 1942, a cat held for rat control died as a result of infection with HPAIV, probably of an antigenic profile equivalent to the previous Japanese A/chicken/Chiba/1/1925 (H7N7) strain. The virus was then isolated from its brain, and it was shown that the Chiba virus antiserum neutralized the virus [44]. Many years later, cats were found naturally infected with HPAIVs in some Asian countries, consequent to eating infected poultry, but there are no further data concerning infection of cats in Japan.

The raccoon (Procyon lotor) constitutes a remarkable example of a North American wild general feeder (27% of his food are vertebrates), of which deliberate introductions and escapes in the mid-20th century, brought about distribution across mainland Europe, Caucasia, and Japan. Since the 1970s, a large number of raccoons have been imported as pets into Japan.

Raccoons routinely travel between wetlands, forests, agricultural operations, and urban and suburban settings. Feral raccoons in Japan were found 0.9% seropositive to HPAI H5N1 virus [45]. More specifically, raccoons in western Japan were likely infected with the clade 2.2 viruses (matching the 2007 HPAI outbreaks in Japan), whereas those in eastern Japan were infected with the clade 2.3.2 virus (matching the 2008 HPAI outbreaks in Japan). Notably, some antibody-positive raccoons in western Japan were captured 6 months before the poultry outbreak with clade 2.2 virus, suggesting that a clade 2.2 subtype H5N1 virus had invaded Japan by 2006.

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Somewhat different findings were obtained elsewhere [46]: urban raccoon sera highly cross-reacted with three HPAI H5N1 strains, namely, clade 2.5: Ck/Yamaguchi/7/04, clade 2.3.2.1: whooper swan/Hokkaido/1/08, and whooper swan/Hamanaka/11, while they displayed a low cross-reactivity with the antisera to the clade 2.2 virus (A/Ck/Miyazaki/K11/2007). However, together with additional subtypes, urban feral raccoons in Japan were found 1.89% seropositivity to H1, H3, H4, H5, N1, N6, and N8. All in all, it is clear that raccoons did contract HPAIVs in Japan, and survived the infection. Alongside, it is not known whether additional HPAIV infections in raccoons took place in Japan, and caused mortality.

Pigs are occasionally infected with HPAIVs circulating in poultry and mallards, and in Indonesia an appreciable infectedness rate with HPAIV H5N1 was found among pigs, without clinical signs [47]. There is no information concerning swine infections with HPAIVs in Japan. Human cases of HPAI did not occur in Japan.

ASSESSING THE OCCURRENCES OF HPAIVs IN JAPAN

Apparent Features

Some of the main above mentioned data pertaining to the occurrences of HPAIVs in Japan since 2003 can be summarized systematically as presented in table 6.

Time of occurrence	Primary infections detected	Secondary infections detected
2003-2004 (H5N1)	Poultry: 28/12/2003 - 3/3/2004	Crows: 22/3/2004-22/4/2004
2007 (H5N1)	Hawk: 4/1/2007	Poultry: 13/1/2007-30/1/2007
2008 (H5N1)	Wild Waterfowl: 21/4/2008 - 15/5/2008	Poultry: None
2010-2011 (H5N1)	Various Wild Birds: 14/10/2010-15/3/2011	Poultry: 15/11/2010-15/3/2011
2014 (H5N8)	Poultry: 13/4/2014-16/4/2014	Wild Birds: None
2014 - 2015 (H5N8)	Wild Waterfowl: 3/11/2014-13/1/2015	Poultry: 15/12/2014-17/1/2015
2016-2017 (H5N6)	Wild Waterfowl: 6/11/2016-24/3/2017	Poultry: 24/11/2016-24/3/2017
2017 (H5N6)	wild waterfowl: 7/11/2017	

Table 6: *Chronologies and hosts of primary and of secondary infections with HPAIVs brought about by virus incursion events into Japan.*

Apart from the past occurrences of H7N7 HPAIVs in Japan in 1925-6 and 1942, there were 6 primary introduction events of HPAIVs, providing an interesting range of variations and versatility, as follows:

- Virus occurrence in poultry only;
- Virus occurrence in wild bird only;
- Initial occurrence not followed by any continual occurrence (during a given introduction event);
- Initial occurrence followed by continual occurrence;
- Initial occurrence in poultry followed by continual occurrence in wild birds;
- Initial occurrence in wild birds followed by continual occurrence in poultry;
- Initial occurrence in clinically infected wild birds;
- Initial occurrence in sub-clinically infected wild birds;
- Initial occurrence in spring;
- Initial occurrence in autumn/winter;
- Involvement of wild birds including waterfowl only;
- Involvement of wild birds including terrestrial birds only;
- Involvement of wild birds including both waterfowl and terrestrial birds;

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- Single-pathway virus introduction;
- Multi-pathway simultaneous virus introductions of one genotype;
- Multi-pathway simultaneous virus introductions of various genotypes;
- Virus introductions from North to South, from South to North, and from East to West.

This wide range of variations and versatility is indicative of transgressions by HPAIVs that can take place in multiple modes leading to virus incursions into Japan, while the principal factor and vector are in all likelihood the movements of migrating birds, even in those cases whereby no migratory birds were found infected in Japan. The reason could plainly be affected, yet undetected migratory birds sojourning in remote places across Japan, or unsampled, subclinically or mildly diseased infected migratory birds. Presumably, such situations naturally brought about, or may bring about additional, undetected HPAIV introductions in Japan. This could readily account, as well, for virus spread from place to place inside Japan, throughout a given introduction event of HPAIV, in those cases during which virus spread over tens or hundreds of kms was unexplained. Multiple secondary introductions into Japan, either of one genotype or more, throughout a one given event of HPAI, could be a plausible alternative explanation. In actuality, both alternatives took place, probably.

Basically, four hypotheses have been suggested for transmission of H5N1 in the HPAI outbreaks in Japan [46]: (1) H5N1-virus-infected chickens may have been imported from other countries; (2) materials (e.g., vehicles and egg containers) from infected area may have been used; (3) viruses may have been carried on clothes, boots, hands, and so forth, and (4) infected wild birds may have carried H5N1 virus into poultry farms to infect chickens.

According to the present analysis, it can be postulated that most, if not all, HPAIV introductions into Japan were brought about by migrating, subclinically infected waterfowl. Presumably, the same applies to spread of HPAIVs inside Japan as well, which was assisted - rarely, yet evidently - by terrestrial wild birds and by blow flies. In that connection, it is of note that the duration of virus persistence throughout a given introduction event varied from few days (the Komamoto isolated event, April 2010), to one month (January 2007), and to maximum of 144 days (autumn plus winter 2010-2011). Spatially (regardless of the Komamoto April 2010 event), virus presence was detected with a minimal distance between the isolates obtained in Towada Lake and additional isolates obtained consecutively in Aomori and Akita (in 2008). In the 2004 event, a distance of only 4 km separated between two isolates from chicken farms in Kyoto, and in that case blow flies have been implicated to constitute mechanical vectors [12], possibly in conjunction with infected crows. By contrast, the maximal distance between consecutively isolated viruses was 670 km, from Kushiro to Fukushima (same genotype, in 2011). Still, considering evident, long-range transfers of HPAIVs by migrating birds across Eurasia during the last decade, such distance could certainly be passed by subclinically infected waterfowl.

The Interfaces with Continental Asian HPAIVs

It so happened that the genesis of the initial index HPAI H5N1 strain A/Goose/Guangdong/1/1996 - an outstanding virus comprising the ancestral origin of all HPAI H5 viruses in Asia - involved four waterfowl viruses, of which two were from Japan - A/Swan/Hokkaido/51/96 (H5N3) and A/Duck/Hokkaido/55/96 (H1N1) - one from Siberia, and one from China.

Since 2002, if not earlier, HPAIVs have become proliferatively endemic in various agricultural areas of East Asia, comprising only H5 viruses, though. The contrast between that fact and the absence of H7 HPAIVs from Asia since 2002 lasted until 2017, when the prevailing LPAI H7N9 turned into HPAI in China. Far beyond, across the vast Siberian territories and north Mongolia, there is an increasing body of evidence pointing at H5 HPAI viruses perpetuation within breeding wild duck populations, each year from April to October. From November to March, after the ducks left, the HPAIVs that were perpetuated by them still have the chance to locally be preserved in the frozen state, thanks to the freezing of lakes - which likely contain HPAIVs - and be contracted once again by waterfowl the next spring, after thawing. If this is the case indeed (as elaborated on and reasoned below), then HPAIVs are perhaps continuously endemic across some wild regions of Siberia, a situation bearing far-reaching consequential implications.

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The occurrence in Japan of the H5N8 HPAIV, with its three genotypes, starting autumn 2014, was a fine exemplification of perpetuating a HPAIV across Siberia throughout spring-summer 2014, and, moreover, of concomitant evolution of three different genotypes from a prototypic H5N8 virus - carried to Siberia by migratory birds from China and/or South Korea - in east, central, and west Siberia, apparently independently of each other. Each of those three genotypes was then carried to Japan and South Korea via different migrating paths, and, in that sense, parallelism to the H5N1 three genotypes isolated in Japan during the 2010-2011 event (as described above) is fairly noticeable.

Collectively, it appears indeed that there are three principal routes, all originating in Siberia, through which AIVs (including HPAIVs), reach Japan:

- From east Siberia, southwards, via Kamchatka, Sakhalin or Primorsky;
- From central Siberia, southeastwards, via Mongolia and South Korea;
- From west Siberia, east-southeastwards, via China, Mongolia and South Korea.

The ecogenetic virus-host interfaces in those three parts of Siberia apparently vary in a way that allows for evolutionary divergence, thereby yielding or shaping different genotypes, while north Mongolia, especially the region of the Mongolian Great Lakes Depression, actually constituting an integral and vital component of central Siberia, in that respect. Further, HPAI occurrences preceding those of 2010 - 2011 and 2014 - 2015 in Japan are compatible with this system, in that the HPAIV occurrence of January 2007 was due to a strain phylogenetically originating from Mongolian and South Korean viruses, while the HPAI occurrence of April 2008 was due to a virus phylogenetically generating the equivalent strain isolated in Primorsky (it is basically assumed that any virus movement that was spatiotemporally traced, is in actuality bi-directional).

Besides, three major migration flyways, each having its typical avifauna, are compatible with the three mentioned Siberian phylogeographic regions, namely, the East Asian-Australasian flyway (East Siberia); the Central Asian flyway (Central Siberia and Mongolia); and the Black Sea-Mediterranean flyway (Western Siberia). Beyond that, though, an additional important route is the west-to-east axis from East China, eastwards, directly or via South Korea, to south-west and central-west Japan, respectively. Those paths presumably underlay the introductions of HPAIVs to Japan in December 2003 and April 2014, and can be regarded as potentially valid courses anytime during the year, not necessarily stemming from seasonal migrations. At any rate, within the East Asian-Australasian flyway, a broader system was identified, notably, including bird migrations through Hong Kong-Guangdong-Shantou Special Economic Zone-Zhejiang-Republic of Korea-Eastern Siberia-Japan; as well as a direct route from Hong Kong to Japan, which appears to be important [49].

On the whole, it may however be observed that the fact that beyond the widespread H5N1 HPAIV, only the H5N8 and H5N6 HPAIVs invaded Japan - is incidental. The chances seem basically equal for those three viruses to reappear in Japan, and for the HPAIVs H5N2, H5N3, and H5N5 found in China to appear in Japan. Obviously, such virus introductions cannot be concretely predicted. Nevertheless, whereas all the mentioned HPAI subtypes continue to circulate in continental East Asia, the incursions into Japan included solely H5N1 during 2003 - 2011, and non-H5N1 subtypes - H5N8 and H5N6 - thereafter, while only one subtype features per event.

The Interfaces with European, Oceanian and American HPAIVs

Although the prime input of HPAIVs is introduced to Japan from continental Asia, interfaces with European, Oceanian and American HPAIVs are, and might be in the future, of further importance as well. Those interfaces are discussed here along the following HPAIV occurrences in Japan.

In autumn-winter 2014, the HPAI H5N8 viruses isolated in Japan and affiliated with Genotype Sub-group G (also named Clade A1, generated in west Siberia) included the following representatives:

- 03 11 2014 A/tundra swan/Shimane/41/2014

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- 18 11 2014 A/duck/Chiba/26-372-48/2014
- 01 12 2014 A/environment/Kagoshima/KU-ngr-H/2014(Kagoshima is the crane site)

Alongside, yet, the earliest HPAI H5N8 viruses of the same genotype isolated from wild and domestic birds outside of Japan included several European strains, in addition to Asian ones, namely:

- 25 09 2014 A/wigeon/Sakha/1/2014 (isolated in East Siberia)
- 04 11 2014 A/turkey/Germany-MV/R2472/2014
- 24 11 2014 A/Eurasian_wigeon/Netherlands/emc-1/2014
- 01 12 2014 A/mallard/South Korea/H1924-6/2014
- 15 12 2014 A/turkey/Italy/14VIR7898-10/2014

The above mentioned clustering of the European plus the Japanese, East Siberian and Korean viruses indicates that they have been derived from a single source population, particularly that this move took place after the last reported cases of the Korean outbreak. Thus, the phylogenetic data and the calculated divergence times show that the evolutionary events responsible for generating the Japanese and European H5N8 strains cases occurred in the summer months in a single location (west Siberia). Also, it has been observed that the H5N8 HPAIVs isolated in Japan most likely derived from wild ducks, rather than from cranes whose flyways were restricted to East Asian countries. Alongside, it appears that the Korean prototype virus had been carried to the Siberian breeding grounds as the Baikal teal migrated north, and then the European and Japanese sequences evolved therein [33].

Phylogenetically, Eurasia represents, at large, a unitary entity of IAVs, distinctively from the North American one. While the mentioned European isolates are affiliated with a genotype that came into being in west Siberia (Genotype G, also named Clade A1) [39] - which is indeed consistent with the amplitude of the Black Sea-Mediterranean flyway zone - the Japanese isolates of that same genotype, together with the East Siberian virus from Yakutia (A/wigeon/Sakha/1/2014) and the South Korean virus (A/mallard/Korea/H1924-6/2014), originated far beyond that amplitude, eastwards. This may be regarded as a solid indication of sound connectivity across the breeding aquatic avifaunas all along the Siberian block, in terms of viral transactions. In that connection, the chronology of obtaining the isolates from infected wild birds, allover (in this and any other case), does not necessarily reflect, obviously, the actual temporal sequence of the pertinent HPAIV occurrences, of which some remain always unsampled. Still, this chronology at times does reflect or is indicative of the real order of phylogeographic HPAIV origination, and should therefore be assessed. Collectively, it means, nonetheless, that various AIVs found in Europe, either ordinary or HP, are basically liable to be carried to East Asia, including Japan, and vice versa. A previous example illustrating such a phylogeographic affinity is the tight clustering of the Japanese and German strains A/duck/Miyagi/47/1977 (H11N1) with A/mallard/Stralsund/416/1981(H2N1) [50].

Still within the Eastern Hemisphere, the East Asian-Australian flyway is of much importance. The plausible introduction into Japan in 2008 of an H5N1 HPAIV by migratory waterfowl heading northwards from Vietnam or Hong Kong to Japan illustrates the transferability of AIVs, both ordinary and HP, throughout this prime flyway, which includes, in addition to East and South-East Asia, Oceania as well. The conveyance of IAVs along this flyway, particularly between Japan and Australia, has been evidenced by Jahangir., *et al*. [51], and by Vijaykrishna., *et al*. [50], with reference to pintail viruses isolated in Japan in Aomori, Miyagi and Akita, and duck viruses isolated in Chiba, Hokkaido and Tsukuba, respectively. Another example in that regard is the close phylogenetic relatedness of the Australian strain A/duck/Australia/749/1980 (H1N1) to the Japanese strain A/duck/Hokkaido/55/1996(H1N1) [53]. Notably, the Australian strain A/shearwater/Australia/1/1972(H6N5) possesses an HA with amino acid sequence most similar to the H6N1 virus isolated from a caged common teal (A/common teal/Hong Kong/W312/1997 (H6N1)) that showed very high (> 98%) nucleotide homology to the HPAI-derived human isolate A/Hong Kong/156/97 (H5N1) in the six internal genes [54].

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Moving to the Western Hemisphere, the role of both Japan and Beringia as springboards for virus conveyance from East Asia to North America is vital, while the related phylogeographic courses are bi-directional. More specifically, it is in fact a triangle, the corners of which are Japan, Alaska and Chukotka, whereby AIV conveyance takes place in all directions, allowing for an ongoing interface with the North American AIV pool. Hokkaido constitutes a main Japanese focus within that context.

Viruses affiliated with Genotype B (Clade A2, East Siberian origin) and exhibiting high homology to the HPAI H5N8 virus isolated in Tottori, Japan, in November 2014, were isolated within one month in the USA Pacific Coast, namely the strains A/gyrfalcon/Washington/41088-6/2014(H5N8) and A/chicken/Oregon/41613-2/2014 (H5N8). Significantly, involvement of a typical migratory waterfowl - northern pintail - was detected in the form of a highly related reassortant virus isolated nearby - A/pintail/Washington/40964/2014(H5N2). In that connection, at the level of a whole virus, conveyance from Eurasia to America has been demonstrated by migrating pintails, with reference to the subtype H9N2 - from China to South Korea, Japan, East Siberia and Alaska [55].

At the level of individual genomic segments, in 38 AIV isolates from pintails sampled in Alaska, nearly half (44.7%) had at least one genomic segment more closely related to Asian than to North American strains [56]. Remarkable inter-hemispheric connectivity underscoring such AIV mobility with respect to Japan, and accounted for by pintails, has been pointed at [51]. Less influential, yet certainly considerably contributory, in that sense, are mallards (which are not so widely migratory as pintails), and additional aquatic birds. All in all, the interface with the North American AIV pool is appreciably extensive and intensive, hence North American inputs of HPAIVs or related gene segments may possibly take place from Alaska to Japan and East Siberia. The probability of such a scenario is nevertheless vague.

Can Local Emergence and Subclinical Circulation of HPAIVs Take Place in Japan?

Within the context of this question, a meaningful factor is the appearance of HPAIV in poultry without there being a preceding virus isolation from a wild bird in Japan, as were the cases in 2003 and in early 2014. This would indicate the feasibility of an undetected HPAIV circulating in wild birds, particularly when the latter are clinically unaffected. Perhaps even more important is the maximal duration of virus persistence in wild waterfowl throughout a given introduction event. It was about 144 days, during the 2010-2011 introduction event. Across such a relatively long period of time, HPAIVs may locally undergo significant evolutionary changes, and resultantly get adapted to some local wild host population, or even give rise to new HPAIV variants, as was repeatedly the case in China. Nevertheless, the ecological conditions in Japan are considerably dissimilar, in various senses, and largely - though not absolutely - restrict virus proliferation.

Unlike other East Asian countries, the component of ducks and geese in Japan poultry is marginal, and this major difference drastically reduces the chances for local emergence or persistence of HPAIVs in Japan. Outbreaks in Muscovy duck farms did happened, but the typically high clinical sensitivity of this host species excludes it as potential HPAIV reservoir. Wild waterfowl presence in Japan is mostly seasonal, and may enable asymptomatic circulation of HPAIVs, mainly within wintering Anas ducks, from November to April. Besides, in principle, during such a period of six months, LP H5 or H7 AIVs may evolve to become HP. Like anywhere, such a pathobiological transition can take place in Japan, although apparently it did not, for the time being.

Beyond that, at least two wild duck species are found in Japan as residents. Across many parts of central and north Japan mallards are present permanently [57]. Also, Mandarin Ducks (*Aix galericulata*) are resident in Japan all year round. Both HPAI H5N1 and H5N8 viruses were isolated in Japan from each of those two wild duck species. Mandarin duck, although not a member of the genus Anas sp., is potentially an asymptomatic carrier, and possibly a perpetuator of HPAIVs, as was the case in a natural infections study specifically referring to this species in South Korea [24]. A comparative experimental infection study of the Japanese isolates A/mandarin duck/Miyazaki/22M-765/2011 and A/chicken/Miyazaki/S4/2011 showed that the mandarin virus possibly adapted to wild birds [27]. Besides, just on a miniature scale, South Japan together with Hokkaido can be viewed as a system which is in principle equivalent, zoogeographically, to South China together with South Siberia. Thus, further to the full residency of mallards and mandarin ducks in Japan, some

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duck species - such as the eastern spot-billed duck (*Anas zonorhyncha*), falcated duck (*Anas falkata*), and tufted duck (*Aythya fuligula*) - migrate in spring from South Japan to Hokkaido and breed therein, thereby staying whole year round in Japan [58]. Potentially, then, they can amplify HPAIV subclinical circulation and perpetuation in Japan.

The populations of wild waterfowl, once infected by a HPAIV, constitute a lasting reservoir for LPAIVs that carry genes previously composing the HPAIV, consequent to reassortments occurring between LPAIVs and HPAIVs. Thus, although scattered, those genes may still retain a potentially unique gene input, which earlier contributed in effect to the virulent pathotype of their ancestral HPAIV, hence could be realized repeatedly through further reassortments events currently taking place, and from time to time generating new HPAI genotypes. The extensive and intensive genomic dynamics marking AIVs in waterfowl at large likely enhances the feasibility of such evolutionary processes.

Non-Anatide aquatic birds, such as cranes, were frequently found infected in Japan; however, they are predominantly migratory, sojourning for about 6 months, and usually do not survive HPAIV infections. Therefore, they can be regarded at most as secondary, short-distance spreaders, but not as perpetuators, of HPAIVs. Basically, the same status applies for the other animals found to harbor HPAIVs in Japan, namely crows, birds of prey, raccoons and blow flies. Nevertheless, the role played by the latter is not negligible. Crows and blow flies contracted the H5N1 virus from diseased or dead chickens (or their feces) during 2004 (in the area of Kyoto). The virus was maintained within the local crow population for several weeks after the outbreak in chickens was over, implying of limited self-sustained virus circulation, though, but whether there were crows that survived infection is not known. Blow flies in all likelihood acted as mechanical vectors. While the virus remains viable for 24 - 48 hours in fly droppings, the in effect duration of virus viability in their intestinal organs is not known.

Predation or scavenging of diseased or dead wild birds probably brought about infection of various birds of prey and of raccoons in Japan. Whereas it may be assumed that birds of prey do not contribute to virus perpetuation, the actual roll of raccoons cannot be estimated, since there is no information about mortality rate and virus transmission among raccoons.

To sum, it appears that HPAIVs can in principle persist in Japan for about six months in wintering waterfowl populations, although in actuality their persistence usually lasted for considerably shorter periods. Potentially, mandarin ducks and mallards, plus several additional wild duck species that are found in Japan permanently may perpetuate HPAIVs for much longer periods, but doubtfully bring about endemicity, due to strict control measures and uncommonness of domestic ducks and geese. Abiotic and semi-biotic factors discussed below as preservers of AIVs are of relevance in Japan, mainly in Hokkaido, and should rather be regarded as contributory to environmental virus persistence. Local emergence of HPAIVs may occur in Japan as anywhere else, in that LP H5 or H7 viruses can transition into HP, unforeseeably. Such LPAIVs are most probably found in Japan, at least from November to May.

DOMESTIC AND WILD WATERFOWL IN CONTINENTAL EAST ASIA AND SIBERIA AS MELTING POTS, RESERVOIRS AND SPREADERS OF SUBCLINICALLY CIRCULATING HPAIVs

Bearing direct, if contrasting, implications for Japan, and constituting a challenging ecoepidemiological issue in itself, this chapter is intended to explore the multi-factorial complexity of HPAIV circulation and maintenance within domestic and wild waterfowl in East Asia and Siberia, in a manner that significantly brings about both spread and endemicity. The factors shaping such capacity are conjunctively biotic and abiotic, having an impact which is largely unique to East Asia, and, consequentially, to Siberia as well. While the component of waterfowl husbandry is typical to East Asia, it is untypical to North America (and Japan). Otherwise, the related, largely influenced, Siberian ecosystem and avifaunas would have been unremarkable in that specific sense, appreciably in similarity to the North American Taiga and Tundra. In that connection, the resistance to - and, resultantly, containment, rather than elimination of - HPAIVs within waterfowl in East Asia and Siberia are common, and widely discussed below, as crucial factors.

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The populations of both domestic and wild waterfowl are important not only in terms of perpetuating HPAIVs, but also in terms of separately retaining the genes of HPAIVs in the following manner. Subsequent to multiple reassortment events, those genes are collaterally positioned in LPAIVs, but may still retain a uniquely potential input - which earlier contributed in effect to the virulent genotype of their ancestral HPAIV - that could be materialized once again due to further reassortments events liable to give rise to new HPAI genotypes. The extensive and intensive dynamics marking AIVs genomes in waterfowl probably allows for such evolutionary courses. Moreover, transitions from LP to HP, and vice versa, can readily happen on the basis of mutational changes only.

Generation and Subclinical Circulation of HPAIVs in Domestic Waterfowl

Conceptual essentials: In the sense of genetic combinatorics per se, both through reassortments and mutations, the chances for generation of HP from LP genotypes in domestic waterfowl are at least equivalent to such eventuality occurring in chickens. Perhaps more significant, then, are the consequent persistence and detectability of HPAIVs, once have they been generated within domestic waterfowl. In case HPAIVs are somehow suppressed within ducks and geese (as elaborated on below), their initial emergence can scarcely be detected. In case they are not suppressed, but cause merely subclinical infections, they might be unsampled, maintained and spread. Those aspects are here looked into, with reference to viruses, hosts and conditions that exist in East Asia.

From 1877 to 1958, a number of epizootics of HPAI occurred in most parts of the world. From 1959 to 1995, the emergence of HPAI viruses was recorded on 15 occasions, but losses were minimal, usually. By contrast, between 1996 and 2008, HPAIVs emerged many times, while four of these outbreaks involved many millions of birds. In reference to recent decades, four phases have been indicated to underlie a paramount shift that took place regarding IAVs ecology [59]:

a) Stable period of LPAI-host interaction.
b) Sporadic transition of H5 and H7 subtypes from LPAI to HPAI.
c) Transient adaptation period of HPAI H5N1 in aquatic poultry.
d) Transient expansion period of HPAI in wild waterfowl.

In other words:

1) HPAI H5N1 viruses of the Gs/Gd-lineage arose in the early 1990s with the ability to persist in geese.

2) Gs/Gd-lineage HPAI H5N1 diversified into sub-lineages and adapted to domestic ducks as host reservoir.

3) Diverse populations of LPAI and HPAI viruses co-circulate in poultry and/or wild waterfowl of Southern China and South East Asia.

4) Gs/Gd-lineage HPAI H5N1 viruses have expanded geographically from China to South East Asia, East Asia, Asia at large, Europe and Africa.

Two HPAIVs, H5N1 and H5N8, seem to best represent the emergence, continuous circulation and dissemination of HPAIVs within waterfowl, thus underscoring the capacities of waterfowl to support the mentioned four phases.

Generation of the H5N1 HPAIVs in domestic waterfowl: A LPAI H5N3 virus isolated from a wild duck in 1991 in Altai Krai - a south Siberian territory, close to the Mongolian Great Lakes Depression - has been pointed at as the primary traceable origin of the first recognized Asian HPAI H5N1 virus, which was isolated from a domestic goose in 1996 in Guangdong, South China, namely AGs/Gd/1/96 [60]. The former virus was probably the progenitor of the Japanese strain A/Swan/Hokkaido/51/96 (H5N3), which contributed a mutated HA to the genesis of A/Gs/Gd/1/96, together with another virus from Hokkaido, A/Duck/Hokkaido/55/96 (H1N1), providing the NA gene, and an earlier Chinese virus, A/Duck/Nanchang/1904/92 (H7N1), providing the six internal GSs [61]. Thus, the index virus A/Gs/Gd/1/96 formed in waterfowl - both wild and domestic - all along the way, involving Siberian, Japanese and Chinese viruses.

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The subsequent evolutionary course of the prototype A/Goose/Gd/1/96 virus involved as well, still, both domestic and wild waterfowl, merely. The virus underwent reassortment events - retaining, yet, its HA GS - and virulently infected chickens, in 1997. Thereby, it acquired an NA GS from a teal virus, namely A/Teal/HK/W312/97 (H6N1), while the donor of the internal GSs was the same teal virus or - apparently less likely - a quail virus, namely A/Quail/HK/G1/97(H9N2) [62]. It later, in 2000, was for the first time isolated from domestic ducks, therein forming a variant genetic group, in parallel to the geese group [63]. Those two basal groups gave rise to a vast variety of H5N1 HPAIVs since then, up to the present, while ongoing virus circulation and evolutionary dynamics - involving multiple duck and geese species, wild, semi-domesticated and fully domesticated - continuously take place in China mainly, in additional Asian countries, and in Siberia. Following is a range of examples, which support this postulation.

Subsequent to the adaptation to ducks of the emerging H5N1 viruses in China, two principal reassortment events occurred in 2001 and 2002, that were responsible for the generation of the majority of 44 distinct Goose/GD genotypes identified by Duan., *et al.* [64] (excepting the 1997 variants). Until 2008, genotype replacement and emergence took place continually, with 34 transient genotypes detected, while of those 10 variants were persistent. Two major replacements of predominant genotypes were also observed: genotype B was replaced by Z in 2002, and then genotype Z was replaced by the now predominant genotype V in 2005. In another study, statistical HA and NA phylogeography of 192 isolates showed that the Chinese province of Guangdong is the source of multiple H5N1 strains, markedly spreading at both regional and international scales [65].

Studies conducted in 2004 in Thailand concretely illustrate the roles played by domestic ducks, with reference to the four systems of duck raising, namely, closed houses with high biosecurity, open houses, free-ranging (grazing) ducks, and backyard ducks. While no infection occurred in the closed houses, the HPAIV was prevalent in the three other systems, and always present in grazing ducks, whereby infection in the hatchery or during the 3 weeks of brooding was detected only after the ducks were released into the rice fields. Although the ducks shed virus for 5 to 10 days, few ducks showed disease signs, and in some flocks no ducks at all were symptomatic [66]. Particularly, wetlands with two cycles of rice crops per year alongside duck production correlate spatially with HPAIV occurrence, because the coexistence of free-grazing ducks and wild waterfowl during a defined period of the year (mainly November to February) may have readily provided an entry point or an index case for HPAI in poultry population in Thailand, and such wetlands apparently act as a HPAIV reservoir [67]. Considering that duck populations remain relatively healthy while excreting sufficient amount of virus to sustain transmission [68], this double crop rice-grazing ducks apparatus may well apply to other countries across East Asia plus Southeast Asia, and hence seems to be of paramount importance.

Additionally to Pekin ducks, two domestic or semi-domestic waterfowl should be mentioned, namely bar-headed goose and green-winged teal. Bar-headed goose might have an appreciable contribution to HPAIV dynamics; its captive rearing and release in China are apparent HPAI H5N1 virus infection routes to wild birds [69], as was probably the case with the important virus A/bar-headed goose/Qinghai/1A/2005. The domestic green-winged teal is a further notable example. It was found in China to be naturally infected subclinically with HPAIVs, such as A/domestic green-winged teal/Hunan/67/2005 (H5N1), a virus that has evident phylogenetic relationships with isolates obtained from chickens, mallards and tree sparrows [70].

Domestic terrestrial hosts, particularly chickens and quails, are obviously involved, chickens usually as dead end, and quails sometimes as generators or amplifiers. Only on rare occasions were viruses from wild terrestrial hosts involved in the generation of new HPAI H5N1 genotypes, such as an isolate obtained from the synanthropic wild bird barn swallow - A/barn swallow/Hong Kong/1161/2010(H5N1) [71].

Generation of the H5N8 HPAIVs in domestic waterfowl: Irrespective of the remarkable evolutionary courses of the H5N1 HPAIVs in Asia, yet still emanating from the HA and PB2 genomic segments of the prototypic strain A/Goose/Guangdong/1/1996 (H5N1) lineage [38], an evolutionary course underlying the phylogenesis of the H5N8 HPAIVs commenced apparently in 2009 in China, took place in domestic waterfowl, foremost, and gave rise in 2014 to a noticeably proliferating virus. More concretely, the generation of the initial

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HPAI A(H5N8) viruses begun in 2009, and progressed through multiple reassortment events, involving, presumably, only duck - both LP subtypes and HP H5N8 strains - in China and Korea, namely:

- A/duck/Jiangxi/28/2009 (H11N9)
- A/duck/Jiangsu/k1203/2010 (H5N8)
- A/duck/Jiangsu/1-15/2011 (H4N2)
- A/duck/Eastern-China/1111/2011 (H4N2)
- A/duck/Zhejiang/W24/2013 (H5N8)
- A/broiler duck/Korea/Gochang1/2014 (H5N8)
- A/duck/Korea/Buan2/2014 (H5N8)
- A/mallard-duck/Korea/W452/2014 (H5N8)
- A/Baikal teal/Korea/Donglim3/2014 (H5N8) [36].

While the three latter Korean viruses - of which two are from wild ducks - possess an identical genotype, the preceding Korean virus (A/broiler duck/Korea/Gochang1/2014 (H5N8)) possesses a different H5N8 genome, but is already HP. The earlier mentioned pre-H5N8 duck viruses came into being in China, during 2009 to 2013. Nonetheless, in China, during 2013 - 2014, a parallel course took place, in this case involving - in addition to viruses from Pekin ducks - viruses from a goose and from a mallard (all HPAIVs), namely:

- A/goose/Jiangsu/WX202/2013 (H5N8)
- A/mallard duck/Shanghai/SH-9/2013 (H5N8)
- A/duck/Beijing/FS01/2013 (H5N8)
- A/duck/Beijing/FS01/2014 (H5N8)

Plausibly, then, a HPAI H5N8 virus reached South Korea for the first time through a mallard moving from China and carrying a virus equivalent to A/mallard duck/Shanghai/SH-9/2013(H5N8).

Along with the five-years evolution of the H5N8 prototype virus, as described, most of the involved precursor viruses were circulating within domestic ducks (and within domestic goose, once), which is a sound indication that those viruses were continuously maintained, and at times possibly substituted, in certain domestic duck populations. It means that such populations serve not only for the genesis of HPAIVs, but also for long-term perpetuation of HPAIVs. In that connection, it has been suggested that the strain A/mallard duck/Korea/W452/2014(H5N8) has the potential to become established in domestic ducks and to alter the genetic evolution of pre-existing avian viruses in poultry, through an interface with the natural AIV gene pool [36]. Such transitions are highly meaningful. Notable as well is the mentioned involvement of the Baikal teal, rather as a virus spreader, in that case.

Generation of further NA subtypes with H5 HA in domestic waterfowl: AAdditionally to the HPAIV H5N8 subtype, the intensive and extensive evolution of H5N1 viruses, particularly within ducks in China, enabled, remarkably, the establishment of a monophyletic H5 clade comprising a variety of novel HPAI H5Nx reassortants that evolved from early members of H5N1 clade 2.3.4 - a group of highly similar H5N1 viruses isolated from ducks in China in 2005. New HPAIVs have emerged that carry H5 proteins capable of matching with multiple NA subtypes. Thus, from 2008 to 2014 H5 HPAIVs surfaced with four different NA subtypes, in healthy Pekin ducks in China, as follows:

- 2008 - H5N5 - A/duck/Guangdong/wy/24/2008
- 2012 - H5N2 - A/duck/Jiangsu/m234/2012
- 2013 - H5N6 - A/duck/Jiangsu/WX156/2013
- 2014 - H5N3 - A/duck/Hunan/?/2014

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Notably, of those subtypes, only the HPAI H5N6 subtype was detected, quite recently, in Japan, although the four subtypes spread over various regions in East Asia, beyond China [72].

Quite exceptionally, domestic muscovy ducks (*Cairina moschata*) were involved in the evolution of the HPAIV H5N6 in Vietnam, through the strain A/Muscovy duck/Vietnam/LBM631/2014(H5N1) [71]. Also, muscovy ducks were found clinically highly sensitive to H5N1 HPAIV experimental infection with the virus A/bar-headed goose/Qinghai/1A/2005, which consistently retained its virulence in this species even after 20 passages [73].

It is of note, that Mexican-like H5N2 vaccines have been used in Hong Kong and other parts of East Asia, from 2003 to 2013. In Taiwan, emerging H5N2 variants were suggestive of possible reintroductions from a vaccine source, and some were developing signs of evolving into a HP strain. Moreover, in Japan, a LP H5N2 subtype virus caused extensive outbreaks in chickens during 2005, and about 5.7 million birds were consequently destroyed. A Japanese ministerial expert committee indicated that the outbreaks might be the result of using an unauthorized defective poultry vaccine that had been illegally imported (apparently from Mexico) [74]. No further use of vaccines against avian influenza took place in Japan thereafter, which probably considerably diminished the prevalence of H5 viruses in Japan at large.

All in all, it is clear that domestic waterfowl, Pekin ducks in particular, plus, secondarily several additional domestic or semi-domestic waterfowl, constitute a cardinal melting pot for ongoing generation and perpetuation of H5 HPAIVs in China, primarily. They play a substantial role in the highly dynamic evolution of those virulent viruses and their delivery across wide areas of East and Southeast Asia. The situation in Japan is totally different, nevertheless, underscoring a huge ecoepidemiological gap from continental Asia, in spite of but a short geographical distance, and a currently imminent virus incursion menace.

Generation and Subclinical Circulation of HPAIVs by Wild Waterfowl

Conceptual essentials: Wild waterfowl represent the chief seeding pool of AIVs at large, including HPAIVs, and, methodologically, the present analysis assumes ecophylogeographic modularity in the following senses:

- Any recognized course/axis of virus movement by means of migrating birds is in principle bi-directional;
- Any recognized course/axis of virus migration potentially pertains to both LPAI and HPAI viruses;
- Any genotype of a present or past avian existent virus could be conveyed, theoretically at least, by certain/various migratory birds from nearly any point to any point worldwide, within a given entire year (or longer duration);
- Nearly any given existent genotype practically can persist perennially - either biotically and/or abiotically (in environmental ice, as detailed below) - regardless of ongoing mutations taking place during biotic phases;
- Upon thawing, nearly any lake or pond is occupied by aquatic birds, worldwide;
- Putative scenarios of genomic reassortment events are basically unlimited, combinatorially, as long as they are not incompatible with empirical findings pointing at certain constrains in that respect, if any (while the survival value of the resultant reassortants is nevertheless another story).

If A and B are the last and the new congregation sites of migratory birds, respectively, then the shorter the distance (hence duration of migration) between A and B is, the higher is the chance for the arrival of still infected waterfowl (that contracted viruses in A) at B, because during flight there is but a little chance for being infected.

China - Hong Kong: *S*ince the globally first detected epizootic of HPAI in wild birds, in 1961 - which yielded the isolate A/Tern/South Africa/1961 H5N3 - such occurrence has not been documented for 41 years, until November 2002, when the evolving Chinese H5N1 virus affected wild ducks, geese, swans and additional aquatic, plus few terrestrial birds in Hong Kong (captive Greater Flamingo (Phoe-

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nicopterus ruber) and other wild birds (Little Egret Egretta garzetta), at two waterfowl parks, and from two dead wild Grey Heron (Ardea cinerea) and a Black-headed Gull (Larus ridibundus), elsewhere in Hong Kong. HPAI H5N1 virus was also isolated from further terrestrial birds, namely dead feral pigeon (Columba livia) and tree sparrow (Passer montanus). Concomitant outbreaks in poultry could not lead to a conclusion as to whether the virus transmitted from wild birds to poultry, or vice versa [75]. At any rate, the 2002 epizootic in Hong Kong - certainly a highly important area of endemic, yet spreadable HPAIVs - unsurprisingly indicated the presence of an increasingly virulent H5N1virus in wild waterfowl, which could thereafter - in cases of subclinical infections and during incubation phases - convey the virus upon migrating northwards.

China - Qinghai Lake: Such virus conveyance, with a major impact, took place three years later. Qinghai Lake, a saline lake, which is the largest lake in China, is where a new, consequential H5N1 lineage formed in 2005, in wild waterfowl. In April 2005, a remarkably extensive epizootic (afflicting more than 6000 birds) occurred in wild species on the remote breeding grounds of Qinghai Lake, north-western China. Infections began in bar-headed geese (*Anser indicus*) soon after their migratory return to the lake, and were followed by infections in great black-headed gulls (*Larus ichthyaetus*), brown-headed gulls (*Larus brunnicephalus*), and great cormorants (*Phalacrocorax carbo*) 10 days later, plus ruddy shelducks (*Tadorna ferruginea*) within 3 weeks. Over half of the reported cases were in bar-headed geese. Experimentally, the prototype virus - A/Bar-headed Goose/Qinghai/0510/05 - was fully lethal to chickens too, and caused 80% mortality in ducks and geese [76]. No epidemiological linkage to poultry could be traced. Named also 'Teal Sea', Lake Qinghai is frequented by teals, which could readily constitute during that episode a totally asymptomatic virus carrier, though, considering that teals are affiliated with the genus Anas sp. In that connection, it has been evidenced that stopover locations used by Bar-headed geese - a common migratory bird along the Chinese-Mongolian major migration axis - are meaningful both for them as well as a number of other important waterbird species; hence a likely mode of conveyance is through a relay effect, namely virus transportation to stopover locations where it can spread among individuals within concentrated areas of feeding and roosting, or through environmental persistence, and then be forwarded to newly infected individuals along the migratory pathway [77]. The latter study identified Qinghai Lake, together with Lhasa, as significant foci of transaction among a variety of wild bird HPAI H5N1 viruses, underscoring the role of wild waterfowl - including bar-headed geese - in medium to long distance spread of those viruses. In addition, it has been postulated that captive rearing and release of bar-headed geese in China is a possible HPAI H5N1 virus infection route to wild birds [69].

Notably, the entire event of the Qinghai Lake 2005 epizootic is indicative of the emergence and spread of a novel HPAI H5N1 genotype within wild waterfowl, in the absence of an interface with poultry, and while no diseased Anas sp. ducks were detected. At any rate, Qinghai Lake is regarded to be a prime nidus and melting pot of AIVs in wild waterfowl and other aquatic birds, at large. Moreover, wide geographic dissemination of the Gs/GD lineage virus was seen between 2005 and 2006, as clade 2.2 viruses spread from Qinghai Lake to Siberia and then to various countries of Asia, Europe, and Africa [39].

Besides, while Qinghai Lake is a major breeding ground for waterfowl in China, it is noticeable that most of the immediate notifications to the OIE about HPAI in China and Hong Kong referred since 2002 to outbreaks in poultry that took place during autumn and winter months - a possible reflection of subclinical virus conveyance by migratory waterfowl reaching China and Hong Kong for the wintering season.

The Mongolian-Siberian domain: North Mongolia provides a proper zoogeographic belt for monitoring intact wild aquatic birds and their pathogens, because wild aquatic birds are very common, while there is very little poultry farming across Mongolia. Therefore, the detection of HPAIVs in resident or migratory birds in North Mongolia would most likely be due to genuine wild birds infectedness, rather than spillover from infected poultry. Basically, the same applies for many areas in Siberia. Recurrent outbreaks of H5N1 in aquatic birds indeed happened across the lakes located in the western and central parts of Northern Mongolia, since 2005, as well as in Siberia, and are here explored, so as to comprehend the unique role of wild waterfowl. Notably, the entire area covers at least 274 migratory waterbird populations of 175 species [78].

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Bridging between China and Siberia - and thus forming, altogether, a cardinal Chinese-Mongolian-Siberian pathway of migratory waterfowl - the land of Siberian Mongolia (namely north Mongolia) may be regarded as a cardinal ecogenetic melting pot of AIVs. Particularly, in the northwest of Mongolia, tucked between the Altai, the Hangayn, and the mountains of the frontier with Russian Siberia, lies a scenic basin complex known as the Great Lakes Depression, in which are strewn more than 300 lakes. The Great Lake Depression is named so because it contains the six major Mongolian lakes: saline Uvs Nuur, Khyargas Nuur and Dörgön Nuur; and freshwater Khar-Us Nuur, Khar Nuur and Airag Nuur. Those lakes, as well as other lakes in the mountainous areas of Northern Mongolia, are abundantly visited by breeding waterfowl during summer. This region appears to constitute a paramount influenza A virus nidus. The mentioned Mongolian lakes are frozen during wintertime - thus acting as possible virus preservers - and the higher (either vertically and/or northwards) their location is, the longer is the longevity of ice, at times perennial, as elaborated on below.

Obviously, the overall AIV gene pool found within wild waterfowl is considerably wider than within domestic waterfowl, thereby facilitating formation of more diversified genotypes, with high evolutionary dynamics. Also, the saline lakes found in northern Mongolia add a further dimension, in that additionally to the waterfowl AIV gene pool, the presence of breeding gulls and terns, as sea-birds, in saline lakes is predominant, with their characteristic AIV gene pool [79]. They considerably enrich and amplify the ecophylogenetic dynamics of IAVs across the region, evidently including the HPAI H5N1 viruses. The largest lake in Mongolia by surface area, Uvs Nuur Lake (saline reservoir), is a key stopover on the Central Asian Flyway during spring and fall migrations. The lake and its feeding rivers are largely occupied by aquatic birds for breeding as well, during spring and summer; so, collectively, it is one of the most important melting pots of AIVs, including HPAIVs, in Asia.

Aside Mongolia, Siberia demonstrated already in 2005 the persistence of HPAI in wild waterfowl, in similarity to the later persistence in Siberia of the HPAI H5N1 and H5N8 viruses that appeared in Japan in 2011 and 2014, respectively (as discussed above). Thus, the appearance in wild birds in Novosibirsk, in June 2005, of a HPAI H5N1 virus affiliated with the Western Siberian sub-group of the 2.2 Lake Qinghai 2005 group, was followed by its spread to Omsk and Altai, and by its perpetuation therein during the whole summer and autumn. The virus was introduced into Siberia by northerly migrating waterfowl through the Dzungarian Gate. Two weeks after its appearance in wild birds in June 2005, it caused outbreaks in poultry in Dovolnoe District - whereby all diseased chickens and turkeys died, yet but partially domestic ducks - and much later, in November 2005, upon autumn migration, it massively and severely afflicted mute swans, for instance in the mouth of the Volga in Astrakhan, an infection that could not be attributed to any poultry source across the area, but to ongoing virus circulating in wild birds sojourning in Siberia until November. Healthy migrating tufted ducks (*Aythya fuligula*) that appeared when the November epizootic started, where probably the carrier [60].

Tuva, representing the Russian Siberian wing of Uvs-Nuur Lake, marked the next detected presence of HPAI H5N1 virus in clinically infected wild birds in Siberia, whereby the strain A/duck/Tuva/01/2006 was isolated in June 2006, affiliated with the Qinghai 2005 group (2.2 clade), once again. One cannot exclude, then, the possibility that the November 2005 virus persisted in Siberia until June 2006. In such case, the virus could have been preserved in the frozen state in Siberia until about April-May, thereafter infecting migratory wild birds upon their arrival at the thawing lakes.

The next HPAIV detected in Siberia appeared in diseased poultry in Far-East Siberia, in April 2008, this time resembling the Fujian 2.3.2. clade, and later, after 14 months, reappeared in June 2009 in clinically infected aquatic birds - no ducks among them - once again in Uvs-Nuur Lake. Many isolates were obtained from gulls and other aquatic birds (like A/black-headed gull/Tyva/115/2009 and A/great crested grebe/Tyva/120/2009), exhibiting high phylogenetic affinity to contemporaneous Mongolian waterfowl clinical isolates of the 2.3.2.1 clade (like A/whooper swan/Mongolia/8/2009, and additional strains isolated from bar-headed geese, ruddy shelduck and common goldeneye; yet not from Anas sp. ducks). No link to poultry, whatsoever, could be traced during 2009 [80], which is indicative of virus conveyance from China by migrating birds and/or ongoing virus perpetuation taking place within the Mongolian-Siberian domain. A similar pattern possibly repeated with the 2011 HPAI H5N1 virus and the 2014 HPAI H5N8 virus, as elaborated on above.

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Virus spreading: Presently, it is completely evident that wild waterfowl are responsible for the spread of HPAIVs over up to thousands of km, either as individual carriers or populations. Two predominant examples are the wide geographic dissemination of the H5N1 Gs/GD lineage virus observed between 2005 and 2006, as clade 2.2 viruses spread from Qinghai Lake, China, to Siberia and then to various countries of Asia, Europe, and Africa [39]; and the conveyance of H5N8 HPAIVs from China to Siberia in 2014, their subsequent extended prevalence throughout Siberia, and their spread to Europe, as well as to America, thereafter. In Siberia, spreading of AIVs is extended during summer by further northerly waterfowl migrations to moulting sites, as long as the northerly moving line of melting lakes and ponds keeps on, until late August. The waterfowl reach almost every melting fresh water body [81]. The same principle applies for HPAIVs tolerably carried by waterfowl.

Throughout East Asia, the current wide prevalence of H5N1 viruses suggests that they may have spread to different countries by the legal and illegal introduction of infected poultry or poultry products. However, it has been fully established that regular movements of infected migratory birds, waterfowl in particular, are an additional substantial factor, if not the prime one. In the East-Asian Australasian and Central Asian flyways, ruddy shelduck (*Tadorna ferruginea*), mallard, common teal, northern pintail, and Eurasian wigeon, are the numerically dominant duck species migrating between breeding areas in Siberia plus Mongolia, and wintering areas in Japan, China, and Southeast Asia, where outbreaks in poultry have been most common [82].

Swans are evidently much more sensitive clinically to HPAIVs than ducks and geese, and therefore do not contribute, usually, to virus spread and perpetuation. Such HPAI victims - rather than carriers - may potentially contribute to spread mainly if they are preyed by other birds or mammals that consequently contract the virus [83]. Even when naturally infected by LPAIVs, swans may be affected, and their foraging and migratory performance are hampered [84]. Migratory geese, and more than that migratory ducks, constitute the main effective spreaders of HPAIVs. On the whole, they convey the viruses all across China, Mongolia and Siberia, as reflected by the major epizootics discussed above.

In relation to Japan, mallards and pintail ducks - as well as additional aquatic migratory birds - are of much importance as spreaders. The autumn migration routes of mallards extend to southern Japan from north-eastern China; to central Japan from south-eastern Siberia; and to Hokkaido from the chain of islands north-east of Hokkaido. Mallards employ a "short travel followed by long-stay at stopover sites" migration strategy. They typically have long stopovers of several weeks between trips of two or three days. Since they have heavy body weight relative to wing area, they need the prolonged staying at stopover sites in order to adequately replenish energy reserves [85]. Therefore, the many stopover sites visited by mallards increase their chances to contract viruses, to shed viruses, and to tolerate infection (due to not getting exhausted, plus due to additional factors, as detailed below).

Alongside mallards, the particular importance of northern pintails as carriers of H5N1 viruses (whether HP or LP) has been evidenced serologically, in Hokkaido, Japan, whereby antibodies to AIV were detected in 64 of 105 samples (61%), while of the 64 positives, 95% inhibited agglutination of H5 AIV antigens (H5N1); it has thereupon been assumed that HPAI H5N1 could contribute to the high seropositivity detected [86]. The precise migrations routes of pintails - and the related gene flow - between China, Japan and eastern Siberia, have meticulously been presented by Tian., *et al.* [49], highlighting the importance of pintails in that sense too. Pintails have indeed been well recognized as a fairly unique AIV carrier between North America and Japan, as well [87,88]. Unlike mallards, though, pintails (and other important HPAIV carriers, such as teals) basically have no direct contact with poultry. It is the mallard who serves as a main, essential bridge, in that regard, between poultry and wild waterfowl, including pintails, teals and more waterfowl. An inter-species barrier that might possibly impede virus transmission among members of the genus Anas sp. doubtfully exists at all.

ATTENUATION AND CONTAINMENT OF HPAIVs WITHIN WATERFOWL

The Benign-Virulent Balance of Virus-Host Interface within Waterfowl

Generally speaking, while infected cells resort to apoptotic death before the assembly of a new viral progeny, the anti-apoptotic measures viruses evolved in order to assure the completion of their full replicative cycle are prominent. Further, viruses may escape neutral-

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ization by host antibodies and may survive a counterattack by the host's T cells directed at infected cells of its own. Thereby, viruses may bring about a form of tolerance and coexist with their host without inducing disease. In its utmost, such a mode takes shape as a fully productive infection, with no cytopathic effect. Persistent and apparently or deceivingly apathogenic or even attenuated viral "quasi-species" populations may contain individual particles that regain virulence due to recombinations and/or gene rearrangements, especially when transgressing species barriers [89]. It appears that at least some of those virus-host interfaces have been adopted by AIVs while infecting waterfowl; and, in conjunction - at times indistinguishably - that waterfowl, particularly ducks, developed attenuating factors leading to the containment of HPAIVs. In general, such coevolutionary paths are characteristic of the interface between viruses and their primal, legitimate hosts. In the case of AIVs, such an interface possibly evolved, initially, within archaic wild ducks. This is the typical situation for AIVs as intestinal pathogens of ducks. However, in terms of heightened virus transmissibility - hence increased survival value of the virus - respiratory shedding might be significantly contributory (beyond intestinal shedding), and is considerably pronounced when duck infection is caused by HPAIV, at times with marked clinical symptoms [91].

Besides, an advantage for a HPAIV being virulent towards waterfowl might be its transmission to other species that prey on diseased or dead waterfowl. Such events have clearly been evidenced with regard to both mammals (domestic cats, and apparently wild raccoons and stone martens) and birds of prey. The peregrine falcon, also known as the duck hawk in North America, is a widespread bird of prey, and was repeatedly found infected with HPAIVs in Japan. Additional infected raptor species in Japan were the mountain hawk-eagle, northern goshawk, grey-faced buzzard and Ural owl.

As presented above, persistence of HPAIVs, H5N1 in particular, in southern China and further areas, since its emergence in 1996, is associated with various risk factors, mainly high densities of domestic ducks raised on rice ponds, and intensively irrigated paddy fields that are also areas which attract wild waterfowl, especially ducks. Conspicuous among the latter are mallards, in that they are affiliated with the same species as Pekin ducks, thus constituting the link of easiest virus transmission to and from Pekin ducks, which mallards tend to naturally approach, alongside with their synanthropic character. Mallards are regarded to be, therefore, the cardinal bridge between poultry and wild avifauna, in that sense. On top of this, their sensitivity to HPAIVs is often - if not normally - sub-clinical, and even if they are affected, the virus might soon shift - due to certain innate properties they possess, as discussed below - into an avirulent form (at least for ducks), still retaining full infectivity and transmissibility.

The wide variability marking the pathobiology of HPAIVs in waterfowl in general, applies to an appreciable degree for ducks, though somewhat differently. Thus, two HPAI H5N1 strains isolated in Japan, namely, A/duck/Yokohama/aq-10/03 and A/chicken/Yamaguchi/7/04, were used for experimental infection of domestic ducks; while the former virus replicated in multiple organs, including the brain, and caused neurological signs, the latter did not cause any clinical signs [91]. The Japanese H5N8 HPAI strain A/chicken/Kumamoto/1-7/2014 caused but subclinical infection in challenged domestic ducks at all doses examined [34].

Domesticated already for thousands of years, Pekin ducks possibly became clinically less resistant to HPAIVs than their ancestral mallards, and such variability - to a certain extent - has been evidenced with several South Korean H5N8 strains. Thus, in experimentally infected domestic ducks, H5N8 viruses were moderately pathogenic (0%-20% mortality rate), while in wild mallard ducks, no severe illness or death occurred at all. A significant difference in the viral shedding was found between domestic ducks and mallards, in that H5N8 Buan2 strain viral titers in domestic ducks were significantly higher than those in mallards on oropharyngeal and cloacal swabs, on 2 and 4 days post infection [92].

Natural infections in South Korea of domestic ducks and of mallards with H5N1 HPAIV showed mortality rates from 0% to 12% in domestic ducks (with strains isolated during 2003 - 2004) [93], and but subclinical infections in mallards (with 2009-2010 strains) [23]. In reference to additional waterfowl species, the H5N1 clade 2.3.2.1 - regarded by the WHO/OIE/FAO H5N1 Evolution Working Group to be particularly associated with virulent wild waterfowl infections [94] - exhibited distinctly high pathogenicity in naturally infected

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waterfowl, for instance in South Korea (2010/2011 winter season), where it was lethal to Baikal teals (genus Anas sp.), spot-billed ducks (genus Anas sp.), mandarin ducks, whooper swans and white-fronted geese; only an infected mallard was found asymptomatic [95].

Natural infections of ducks with different genotypes in China varied pathobiologically; thus, the strain A/duck/Hubei/Hang-mei01/2006 (Fujian clade 2.3.2), infecting domestic ducks in Central China in 2006, was neurovirulent and considerably lethal [96], whereas apparently healthy migratory wintering ducks sampled at Poyang Lake (South-East China) in 2005 shed HPAI H5N1 viruses of genotypes Z and V, including reassortants containing different combinations of all gene segments of the Qinghai-lineage H5N1 [97]. Alongside, active and passive surveillance of wild birds carried out in 14 provinces of China between 2004 and 2007 led to a fairly comprehensive picture. Widely varying levels of HPAI H5N1 prevalence were detected in different bird species, with Anseriformes, particularly subclinically infected mallard ducks, exhibiting the highest incidence of 4.37%. Other species - specifically tufted duck, bar-headed goose, and whooper swan - occasionally, yet rarely, showed obvious sign of disease or death [98].

In general, clinical sensitivity to HPAIVs clearly increases from ducks to geese, and further to swans, although all are Anatide. In two major HPAI episodes - Guangdong 1996 and Qinghai 2005 - diseased geese were predominant, yielding the index viruses. Moreover, there is apparently decreasing clinical sensitivity from ducks not affiliated with the genus *Anas* sp., to ducks of this genus, and particularly mallards. For example, in July 2009, HPAI H5N1 viruses of clade 2.3.2.1 were detected in dead and sick migratory waterfowl in Mongolia (whooper swans, bar-headed geese, ruddy shelduck and common goldeneye) and in neighboring Siberia (black-headed gull, spoon-bill, great crested grebe, and little grebe). By contrast, as one instance, of note are the subclinical infections of Tundra swans that introduced the HPAIV H5N8 into Japan in 2014, on the one hand, and the lethal infections of mallards that later on contracted the HPAI H5N8 virus in Japan, on the other hand. Still, the Tundra swan is the smallest swan in the Northern Hemisphere, and rather resembles a goose [99]. Experimentally, several other swan species, black swans in particular, were found extremely affected by HPAI H5N1 virus challenge, in comparison to geese, yet virus shedding did take place sometimes before disease appeared [100].

Subclinical Persistence Mechanisms of HPAIVs in Ducks

A comparative experimental infection study was conducted with H5N1 HPAIV in mallards and Pekin ducks, too. It was thus shown that there is no substantial difference in the outcome of HPAI H5N1 infection in mallards and Pekin ducks, yet a slight increase in disease progression and higher intravenous pathogenicity index scores for HP viruses, observed in Pekin ducks, may indicate a somewhat higher clinical susceptibility in this domestic sub-species. At any rate, it has thereby been postulated that H5N1 pathogenicity in ducks does not correlate with viral genotypes, and may be due to genetic traits more subtle than genotypes, such as an individual allogene, or even just a few specific amino acids [101]. In that connection, the two Japanese wild duck H5N1 HPAIV isolates A/duck/Hokkaido/WZ83/2010 and A/duck/Hokkaido/WZ101/2010 were used for experimental infection of domestic ducks, demonstrating that a single amino acid difference at position 43 of the M1 gene particularly affected viral pathogenicity in challenged domestic ducks, and much less in chickens [102].

Also, it has been evidenced that in domestic ducks challenged with the H5N1 viruses isolated in 2004, diminishing pathogenicity allowed for the shedding of detectable virus for long periods (facilitating virus transmission), and variant viruses were selected within a single passage; these H5N1 viruses became less pathogenic to domestic ducks but remained pathogenic to other domestic poultry. Remarkably, however, two virus sub-populations, one LP (for ducks, but HP for chickens), and the other one HP (for both ducks and chickens) were revealed, while an equilibrium has been retained between them [68]. By contrast, a domestic duck not affiliated with *Anas* sp. - muscovy duck (*Cairina moschata*) - was clinically found highly and persistently sensitive to H5N1 HPAIV A/bar-headed goose/Qinghai/1A/2005 challenge, and the virus retained its virulence even after 20 passages [73].

Thus, it appears that ducks (mainly of the genus Anas sp.; mallards and Pekin ducks in particular) have a capacity to bring about attenuation of HPAIVs, at least partially or temporarily, in the form of the mentioned two sub-populations. Such an equilibrium is a key parameter, considering that it is sometimes not clear whether an eventual transition from low to high pathogenicity takes place during a

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certain viral passage just randomly and independently of the preceding passages, or whether the transition is a gradually gathering one. At times, only one or very few critical mutations are needed, at times many mutations are needed, and at times just one genetic reassortment event is needed. The duration of the transition from LP to HP appreciably varies, and frequently gives rise to intermediate strains that actually cannot be classified as LP or HP viruses. As one instance, many LPAI and HPAI strains of the subtype H5 (and H7) exhibit inconsistency in terms of correlation between a deletion in their NA stalk and their virulence [103].

Huang., *et al*. highlighted genetically optimized defense mechanisms against influenza infection of Anas platyrhynchos, where the optimized immune system can be overcome by a HP H5N1 virus (A/duck/Hubei/49/05), but not by a LP H5N1 virus (A/goose/Hubei/65/05), a distinction which identifies disruptions in the long-standing equilibrium between this key host species and AIVs [104]; yet the very fact that HPAIVs often cannot overcome the optimized immune system in this species indicates tolerance and containment earned through prolonged evolution.

By obtaining miRNA repertoires and expression levels in the three major immune organs of Pekin ducks and chickens, namely, the spleen, thymus and bursa of Fabricius, in the presence or absence of H5N1 HPAIV infection (with A/Duck/Anhui/1/2006), it was found that in dissimilarity to chickens, no obvious changes in the gross lesions in the duck immune organs were observed. The divergent miRNA expression changes of infected chickens and ducks reflected the different status of infection, and were regarded to have great impact on the expression of target genes in pathways related to immune response, hence on the pathogenicity of the virus [105]. However, previously acquired immunity probably plays a role. It was found that mallards with prior exposure to homologous LPAI viruses may remain healthy and might be suitable for long-distance transposition of HPAIV, but probably only shed very low titers of virus, while mallards with prior exposure to heterosubtypic LPAI viruses might pose a greater risk for transmission and spread of HPAIV, because they can shed higher amounts of virus (but only via the respiratory route), without developing severe clinical disease [106].

Pekin ducks infected experimentally with LPAIV A/mallard/BC/500/05 (H5N2) and with HPAIV A/Vietnam/1203/04 (H5N1) initiated a minimal response to the LP strain, yet an immediate and robust response to the potentially lethal strain, while major histocompatibility complex class I, interferon induced protein with tricopeptide repeats 5, and 2′-5′oligoadenylate synthetase-like gene were considerably increased in relative transcript abundance in duck lung [107]. Multi-factorial mechanisms seem, thus, to typically underlie HPAIV attenuation. Further, the RIG-I - a cytoplasmic RNA influenza virus sensor, which is found in ducks (but not in chickens) - plays a role in clearing an influenza infection, and may reflect the differential susceptibility of ducks and chickens to influenza-induced pathology. It was shown that RIG-I is expressed during the innate immune response to influenza infection in ducks, providing evidence for the antiviral relevance of RIG-I in the natural host of the virus [108].

Pekin ducks infected experimentally, yet subclinically, with the HPAI virus A/turkey/Italy/4580/99 H7N1 initiated a fast but low cytokine response followed by the activation of major pattern recognition receptors (RIG-I, TLR7, MDA5) and a persistent cellular immune response, whereas in chickens, excessive delayed cytokine inflammatory responses but inadequate cellular immune responses probably contributed to intensive pathogenetic effects [109].

An additional factor - signal transducer and activator of transcription-3 (STAT-3) - has been pointed at. It is likely that excessive proinflammatory response in H5N1 HPAI virus infected chickens could be mediated through the inhibition of STAT-3, whereas a functional STAT-3 corresponds to an attenuated pro-inflammation in H5N1 virus infected ducks [110].

Epigenetic mechanisms involving host DNA methyltransferases were revealed during experimental infection of various human tissue cultures with the Chinese HPAIV H5N1 virus A/chicken/Hubei/327/2004. The loss of methylation at the promoter region enhanced the transcript factor binding, which in turn activated expression of proinflammatory cytokines, thereby increasing pathogenesis and exhibiting anti-influenza activity. Both the influenza virus and its replicative intermediate dsRNA activated cytokine promoters, increased their transcription, and enhanced cytokine secretion [111,112]. Conceivably, a counter epigenetic mechanism operates during infection of ducks with HPAI H5N1 viruses, ultimately leading to an opposite outcome, namely, virus attenuation and containment. Notably, epigenetics represents functionally relevant alterations in the genome that do not involve a change in the nucleotide sequence. In terms of virus-host modular biology and consequent evolutionary plasticity, the described epigenetic alterations related to HPAIV take place in the host cells and not in the virus particles, and are not inherited; but the ability or inability to induce them is a cardinal viral trait, shaped by the viral genome.

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Collectively, it is clear that the species *Anas platyrhynchos* (both wild and domestic) - and, sensibly, the genus Anas sp., at large - possesses, either genetically, epigenetically, and/or otherwise, certain properties that support attenuating and perpetuating HPAIVs. And the implication is a flexible mode of duck infection thus formed, which would favorably (for both the virus and the host) generate a non-immunogenic, fully productive, and yet tolerable infection. Favorably as well, yet, moderate immune response would limit infection to a level still enabling subsequent viral transmission, and leave the host alive and liable to be infected by antigenically different virus variants. Basically, this is expected for a primal host species (mallard), which conceivably might have been adapted to infections with AIVs in such an innately regulated mode since its archaic evolutionary era. Such a host-virus interface has nevertheless immense consequences at present, regarding the ecology of HPAIVs, their lasting perpetuation, and their epizootical impacts, particularly during the last 20 years, across East-Asia and Siberia.

VIRUS PRESERVATION AND DISPERSAL DUE TO SEMI-BIOTIC AND ABIOTIC FACTORS

Environmental Multi-Month virus Preservation and Dispersal

Semi-biotic and abiotic factors constitute essential complementary facilitators to biotic factors (various infected hosts), which allow for the continuous preservation and dispersal of HPAIVs, especially in frosty geographical belts of Eurasia and North America, where during winter no waterfowl are found. The persistence of AIVs in duck feces, duck carcasses and lake sediments is well known, and was found to be significantly prolonged, particularly for duck feces and lake sediments at 10 and 0 degrees C (tens to hundreds of days) [113]. Such temperatures are typical of Siberia, Mongolia and Hokkaido. Less known are the roles played within that context by feathers, ice-water transitions, and winds, hence the following discussion is elaborative in those respects.

The Japanese HPAI H5N1 strain A/Chicken/Yamaguchi/7/2004 was found to have a predilection for feathers in challenged domestic ducks [114]. Also, the persistence in detached feathers of two H5N1 HPAIVs of different genotypes isolated in Japan - A/chicken/Miyazaki/K11/2007 and A/whooper swan/Akita/1/2008 - was studied subsequent to intranasal challenge of domestic ducks. Viral infectivity persisted in the feathers for 160 days at 4 degrees C, and for 15 days at 20 degrees C. Viral titers of 10(4.3) 50% egg infectious doses/ml or greater were detected for 120 days in feathers stored at 4 degrees C [115]. Further, it has been demonstrated that feathers covered with preen oil could efficiently capture and concentrate AIVs from water [116]. Resultantly, the virus particles adsorbed on bird's bodies may mediate infection through self-preening or allo-preening activities. Although feather swabs collected from experimentally preened mallards were positive by virus isolation roughly for one month [117], it is not known how long the virus can survive in preened feathers in nature. Nagy., *et al*. speculated the possibility that the hydrophobic preen oil on detached feathers provides a sufficiently protecting environment for between-year persistence [118]. This appears to be feasible, considering the presence of the lipid bilayer envelope of IAV, of which the protection lent by may be enhanced through the hydrophobic preen oil. At any rate, those studies clearly show that detached infected feathers of waterfowl can play an important role, in terms of semi-biotic or abiotic perpetuation of HPAIVs in the environment. During wintertime, in Siberia, Mongolia and Hokkaido, feathers may thus act as preservers of HPAIVs (for more than 5 months). In that connection, it is of note that waterfowl have their molting season right after the breeding season, in various northern areas of Siberia.

Lake water, either in the liquid or frozen state, appears to be the natural abiotic reservoir of AIVs. Thus, Qinghai Lake, for instance, often remains frozen for three months continuously in winter. In Qinghai Lake water - which is salty and alkaline - at 4°C, infectivity of H5N1 could be maintained for 23 days, whereas in Poyang Lake water - the largest freshwater lake in China - at 4°C, infectivity of H5N1 could be maintained for 43 days [119]. In general, AIV viability normally decreases as water salinity or temperature increases. At any rate, such appreciable duration of virus persistence enables continuous chain of infections at the level of the avian population occupying the water, hence continuous presence in water of viruses shed by the pertinent population.

Environmental Multi-Year virus Preservation and Dispersal

It has been observed [120] that abiotic environmental preservation of AIVs ought to regularly take place for periods of 2 years at least, due to: offering a parsimonious explanation of the 2 - 4-year periodicity of avian influenza epidemics; provision of a virus persistence mechanism within small communities where epidemics cannot be sustained by direct transmission only (i.e., communities smaller than the critical community size); and the very low levels of environmental transmission (i.e., few cases per year) that are sufficient for avian

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influenza to endure within populations where it would otherwise vanish. In that connection, the ecogeographic, biophysical and genetic feasibility of multi-year AIVs preservation in environmental ice has been elucidated [121]. Briefly, such feasibility is based on the following postulations:

1) The virions can survive in lake water during the relatively short period of time between ice thawing and refreezing, in sub-arctic and arctic regions.

2) Biophysically, the virions are capable to:
   - Survive freezing;
   - Persist viably in the frozen state until thawing occurs next year (or later);
   - Survive thawing;
   - Persist again in lake water until recontracted by waterfowl.

3) Portions of the virion population survive repeated cycles of freezing and thawing until eventually recontracted by waterfowl.

In arctic and sub-arctic regions, water bodies are frozen, entirely or partially, for 4 months (in the southern Taiga) up to 10 months (in the northern Tundra and Arctic Ocean islands), annually, in the form of seasonal ice. Most water bodies are occupied by waterfowl whenever thawed, and are thereby seeded with AIVs. This is the case across Siberia, entirely. Perennially frozen lakes are found in islands located in the Arctic Ocean, extending, thus, the range of the waterfowl northerly migration axes, in that whenever thawed and until refreezing occurs, those lakes are occupied by breeding aquatic birds, which are prone to contract viable influenza viruses released by melting ice. As elaborated on above, North Mongolia may be regarded as a pristine intact ecosystem that constitutes a prime genetic melting pot of IAVs, AIV in particular, for thousands - if not many more - of years. In principle, the same cryobiological apparatus operates across most of the Siberian territories.

Further, HPAI-infected wild birds present across below-zero-geographic belts might die because of the infection or just freeze to death. In such cases, which may not at all be rare, virus preservation in the frozen state will probably take place until the carcasses are eaten or defrost. The principal feasibility of such scenarios has been illustrated in 2007 in Europe, whereupon backyard chickens that had access to uncooked offal from commercial deep-frozen duck carcasses contracted HPAI H5N1 viruses, thus propelling wide outbreaks [122].

Irrespective of preservation in the frozen state, waterfowl often occupy rivers and streams, in addition to lakes and ponds, hence water currents may readily play a role in spreading HPAIVs. Air and dust currents apparently constitute a further abiotic contributor. H5N1 outbreaks in downwind areas of Asian dust storms suggest that viruses might as well be transported by dust storms, which are becoming across Asia longer and more frequent as a result of desertification in China; viruses attached to particles of dust stirred up by these storms can thereby potentially travel long distances and occasionally reach far locations [123]. In Europe, the contribution of a possible wind-mediated mechanism to the total amount of spread during the 2003 H7N7 HPAI epizootic (in the Netherlands) has been estimated to be around 18% [124].

CONCLUSIONS

The present study provides a rare opportunity to inquire into the pathobiological profile, the epidemiological profile, and the related ecophylogeographic mechanisms marking a viral pathogen - AIVs - that is both waterborne and airborne, tolerated and carried for long distances by migratory waterfowl in its both benign and virulent forms, fairly prevalent in those two forms across various parts of continental East Asia, and sustains a dynamic, yet infrequently virulent interface towards Japan.

Physically detached from continental Asia, and adhered to a strict HPAI monitoring regime, Japan enables an intact investigation of that interface. Thus, while a variety of AIVs are currently conveyed to Japan by migrating aquatic birds, HPAIVs are occasionally found among them, in particular when the origin is China, Mongolia or Siberia. Those three regions, or certain parts of them, appear to comprise the main cradles of HPAIVs in Asia, and, at the same time, the effectual springboards for the dissemination of those viruses across East Asia, at times including Japan. Dissemination to Europe and to North America has been fully evidenced as well.

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Basically, Japan is appreciably vulnerable within that context. Constituting the eastern edge of Asia, right before the Pacific Ocean, it is occupied as the final stop by plenty of migratory birds, of which many are aquatic birds that reside in the long coast lines of Japan, and in the lakes found in Japan. Potential, subclinically infected carriers of HPAIVs are not rare among them. Nevertheless, during the period that followed the first detection of HPAIV in Japan, namely from 1927 until present, only seven primary introductions of such viruses where detected in Japan, during 2003 - 2017. It may be assumed these data imply that additional introductions of HPAIVs into Japan did occur, and were not detected, while a reasonable scenario is presence of infected wild waterfowl that were not at all diseased, or were diseased but fully recovered. Such scenarios are in principle most feasible, and if the virus is not transmitted to poultry or to clinically highly sensitive wild birds, there is considerable chance for undetected virus presence. As long as this mode persists, virus circulation may thereby continue undetected, until the infected wild waterfowl have their next migration off Japan. No consequent impact, whatsoever, is expected during such eventualities, unless the pertinent HPAIV is contracted and perpetuated by wild ducks that are resident in Japan, namely mandarin and mallard ducks, foremost. Those two species are apparently the main potential reservoirs of HPAIVs in Japan, and were found infected with HPAIVs in Japan in actuality.

Otherwise, the possibility that viruses will be contracted and perpetuated by domestic ducks, or geese, seems to be faint, due to the uncommonness of domestic waterfowl in Japan. The rarity of domestic ducks and geese in Japan, particularly in comparison to their abundance in continental East Asia, is a cardinal advantage Japan has, in addition to its position as an island, and the rigorousness of implementing biosecurity regulations. The combination of those three factors is in all likelihood of crucial importance for the infrequent presence of HPAIVs in Japan. Notable, however, is the 2003-2004 H5N1 introduction event, as well as the early 2014 H5N8 introduction event, whereby no detected wild waterfowl were involved on the one hand, while alongside repeated outbreaks in chicken farms, direct involvement of infected crows and blow flies was evidenced, on the other hand, during the 2003-2004 episode. Infected birds of prey were at times found in Japan, but their epidemiological role appears to be marginal, if any. Nearly unique to Japan, across Asia, is the contraction of HPAIVs by raccoons, although no indication exists as to their actual contribution to HPAIVs circulation in Japan.

At any rate, an intriguing issue is the modes of long-lasting HPAIV perpetuation throughout China - probably by both domestic and wild ducks and geese - plus Mongolia and Siberia - probably by wild waterfowl, foremost. The key species within that context is evidently *Anas platyrhynchos* (mallards and Pekin ducks), followed by other *Anas* species (like pintails and teals), additional duck genera, and certain wild geese. Ducks - plausibly the primal host of AIVs at large - most likely maintain a singular interface with HPAIVs, in that they - in conjunction with certain viral attributes - have the capacity to attenuate and perpetuate HPAIVs. This outstanding capacity, which is elaborated on and discussed above, embodies a critical factor in HPAIVs ecoepidemiology. It is apparently augmented by semi-biotic and abiotic enduring elements, like infected feathers, frozen feces, lake water, and lake ice, which may act as lasting environmental virus preservers. Their contribution is rather more pronounced and vital in Mongolia and Siberia, due to the frosty temperatures prevailing across those two territories from December to May, while waterfowl are wintering elsewhere. HPAIVs may thus be preserved in the environment until waterfowl return next spring and be contracted by them, commencing renewed biotic virus circulation.

Ongoing virus perpetuation and biotic circulation facilitate the highly dynamic processes marking the evolution of HPAIVs in China, Mongolia and Siberia, as intactly analyzed in the present study, in regard to the various HPAIVs that invaded Japan. It has thereupon been shown that clear - at times intriguingly complex - phylogenetic affinities exist between all strains of both H5N1, H5N8 and H5N6 subtypes isolated in Japan and certain isolates from China, Mongolia and Siberia, plus nearby South Korea. The related phylogeographic and phylochronological profiles constructed thereby underscore a coherent evolutionary system that has direct interfaces with Japan, and thus bears significant epidemiological implications. Secondarily, but largely extended spatially, phylogenetic affinities emerge among Japanese, European, American and Oceanian HPAIVs, adding a further interesting dimension.

All in all, the presented data and analyses highlight in detail a distinct, remarkably broad ecoepidemiological and ecophylogeographic machinery, which is propelled and fueled by intensive interfaces existing between continental Asia and Japan, and yet accentuates major

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and meaningful differences between those two regions, in regard to the generation, occurrence and persistence of HPAIVs. The sharp contrast illuminated thereupon poses two interacting, substantially dissimilar systems, albeit very close spatially to each other, across which the virological, pathobiological, and evolutionary dynamics of HPAIVs is consequentially well illustrated.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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