

## CHAPTER 3

# H5N1 HPAI IN DIFFERENT SPECIES

This chapter reviews the role of various species in the genesis, persistence and spread of Asian-lineage H5N1 HPAI viruses. Special attention is paid to the domestic duck (*Anas platyrhynchos* var. *domesticus*), which is considered to have played a key role in these processes. An understanding of the role of domestic ducks in the maintenance and spread of these viruses helps to explain why the current outbreak of HPAI is panzootic whereas earlier outbreaks were not.

### 3.1 H5N1 HPAI AND DOMESTIC DUCKS

Ducks (wild and domestic) are capable of being infected subclinically with a wide range of avian influenza viruses but until recently most isolates from ducks were of low pathogenicity (see for example Stallknecht *et al*, 1990b).

Prior to the emergence of Asian-lineage H5N1 HPAI viruses there were few reports of natural infection with HPAI viruses in ducks. One noteworthy exception occurred in Ireland in 1984, involving an HPAI virus of the H5N8 subtype. This highly pathogenic virus was detected in clinically normal domestic ducks on a farm adjacent to a turkey farm that several months earlier had experienced outbreaks of HPAI (Alexander *et al*, 1987).

Experimental infections of ducks with HPAI viruses (other than Asian-lineage H5N1 HPAI viruses) have yielded mixed results. In one study, ducks were relatively resistant to experimental challenge to a range of HPAI viruses (Alexander *et al*, 1978), although other studies found that ducks could be infected subclinically. Despite the lack of clinical signs, some of these infections were systemic (see for example Kawaoka *et al*, 1987; Nestorowicz *et al*, 1987). These experimental studies and the earlier field experience in Ireland demonstrated the potential for clinically normal but infected ducks to propagate and spread HPAI viruses.

Studies conducted in Hong Kong prior to 1997 did not lead to detection of avian influenza viruses of the H5N1 subtype in domestic ducks, suggesting that the emergence of Asian-lineage H5N1 HPAI viruses is a relatively recent event. Over a five-year period, between 1975 and 1980, samples were collected from domestic ducks (8 737), geese (1 353), and chickens (1 708) originating from southern China and Hong Kong. A total of 586 avian influenza viral isolates were obtained. Of these, 22 were H5N3 subtype (21 from ducks and one from a goose), and one (from a duck) was an H5N2 subtype (Shortridge, 1992). These H5 viruses were not direct precursors of the Asian-lineage H5N1 HPAI viruses.

The first recorded cases of infection with H5N1 HPAI viruses in domestic ducks in Asia were in 1997 from live-bird markets in Hong Kong SAR (Shortridge, 1999). These positive samples were collected in late December 1997, at a time when levels of infection in terrestrial poultry in markets were extremely high. It is possible that these cases resulted from spillover of infection from terrestrial poultry back to ducks, rather than ducks being the initial source of the virus for the chickens (the percentage of chicken samples yielding virus was far greater than the percentage of positive samples from ducks). This was supported by subsequent experimental infection with a 1997 H5N1 virus isolate from Hong Kong SAR (HK/97 strains) which indicated that ducks were relatively resistant to infection with HK/97 strains of virus, although virus was recovered from oropharyngeal swabs and from the lung of one inoculated duck (Shortridge *et al*, 1998; Perkins and Swayne, 2002). This suggested ducks played a minimal role in the

perpetuation of the 1997 Hong Kong SAR H5N1 HPAI viruses. Once these viruses were eliminated from Hong Kong SAR in 1997, no H5N1 viruses with the same combination of internal genes and NA gene (HK/97-like viruses) were detected in imported ducks (or indeed other poultry), despite regular intensive surveillance.

The first case of infection with an H5N1 HPAI virus in domestic ducks in mainland China was not reported until 1999 (Chen *et al*, 2004). This sample was reputedly from a healthy duck on a farm in Guangxi Autonomous Region. Seven of the eight genes of this virus were Goose/Guangdong/96-like but the virus had acquired a different NS gene from an unknown source.

By late 2000, healthy ducks transported to Hong Kong SAR from mainland China were found to be infected with H5N1 HPAI viruses (Guan *et al*, 2002a). These viruses differed from those seen previously in that they were reassortants, having acquired internal genes from other influenza viruses, presumably from aquatic birds. This was the first indication that apparently healthy ducks excreting H5N1 HPAI viruses could spread infection across territorial boundaries.

The first reported case of infection with a so-called 'Z' genotype H5N1 virus was apparently from a normal farmed duck in 2001, also in Guangxi Autonomous Region (Sims *et al*, 2005). In an earlier publication (Chen *et al*, 2004) this was referred to as a 'G' genotype virus.

In 2001, an H5N1 virus was detected in meat from a duck imported to the Republic of Korea from China (Tumpey *et al*, 2002). This case again demonstrated that ducks healthy enough to be presented for slaughter could be systemically infected, and also showed the potential for long-distance transport of virus through trade in poultry meat. Subclinical systemic infection with H5N1 HPAI viruses was confirmed through experimental inoculation of susceptible ducklings (Tumpey *et al*, 2002). The virus was capable of replication in many tissues of inoculated ducks, including muscle and brain. Compared with some earlier H5N1 HPAI viruses isolated from poultry it multiplied to higher titres in lung and kidney.

Fatal disease in ducks caused by H5N1 HPAI viruses was not reported until 2002-03 and involved Anatidae (and other orders) in two zoological collections in Hong Kong SAR. This involved two separate genotypes (Z and Z+) suggesting different origins for these two outbreaks, which were located some 12 km apart (Ellis *et al*, 2004; Sturm-Ramirez *et al*, 2004).

Studies in Thailand in 2004 demonstrated that, under field conditions, not all infected ducks developed signs of disease (Songserm *et al*, 2006). Experiments conducted elsewhere demonstrated that the outcome of inoculation depended on the strain of virus (Sturm-Ramirez *et al*, 2005) although specific molecular changes associated with the increased capacity to produce disease in ducks were not determined. Others have demonstrated an age-related difference in susceptibility (Swayne and Pantin-Jackwood, 2006; Pantin-Jackwood and Swayne, 2007).

In ten flocks of grazing ducks in Thailand that were examined in detail, infection was not detected while ducklings were being brooded but occurred within 12 to 63 days after the birds were released for grazing. It was not determined how these ducks became infected (Songserm *et al*, 2006).

The detection of infection in subclinically infected ducks in Thailand was achieved through intensive targeted surveillance. Enhanced surveillance starting in October 2004 included apparently healthy ducks and led to a marked increase in the number of cases detected (Tiensin *et al*, 2005).

Ducks are now known to be capable of excreting H5N1 HPAI virus via the cloacal and respiratory routes for at least 17 days (Hulse-Post *et al*, 2005). During this time, if they contact other poultry either directly or indirectly, they could potentially spread infection, assuming the quantity of virus excreted exceeds the infectious dose for the exposed poultry.

Recent studies have demonstrated the presence of virus in feather epithelium of call ducks experimentally infected with H5N1 HPAI virus, suggesting that shed feathers could pose a potential risk for spread of infection (Yamamoto *et al*, 2007).

### 3.1.1 Other relevant information on management and marketing of ducks

The numbers of ducks reared in China increased more than three-fold from a standing population of 223 million in 1985 to 725 million in 2005 (FAO, 2006) with many of these reared on ponds, potentially allowing contact with wild birds. This increase in the number of domestic ducks increased the number of susceptible poultry in which H5N1 viruses could multiply and, given the predominance of live-bird marketing and lack of segregation of species, probably increased the chance of spread of these viruses to other poultry and humans. Recently, this susceptibility has been reduced to some extent through the use of vaccination and, in some places, through segregation of terrestrial and aquatic poultry in markets and during transportation.

The number of ducks in Viet Nam also increased in the latter part of the 20<sup>th</sup> century and early 21<sup>st</sup> century (until numbers fell in 2004-05 as a result of HPAI control measures, including breeding bans) but not to the extent seen in China (approximately four percent annual growth over the previous nine years) (FAO, 2006). Most of these ducks are reared on channels, ponds and paddy fields with very few reared on enclosed intensive farms.

China and Viet Nam account for 75 percent of the world's duck population (about 775 million of the approximate standing population of 1.044 billion (FAO 2006). Both Indonesia (around 34 million) and Thailand (around 17 million) also have relatively large duck populations. Overall, approximately 90 percent of the world's domestic ducks are in Asia (computed from FAO, 2006).

Ducks are relatively high value animals and are transported over long distances to markets. For example, in China it is known that they travel more than 400 km from inland provinces such as Hunan to coastal markets in Guangdong (see for example Li *et al*, 2004a).

Grazing of ducks on paddy fields has been practised for many years but the development of a specific grazing industry involving the long-distance trucking of ducks across Thailand is a relatively recent phenomenon. Until about 30 years ago, this was apparently a local practice with limited movement of ducks across provincial boundaries (D. Hoffman, personal communication).

Most ducks in Asia are reared extensively and this is considered to be a key factor in the transmission of H5N1 HPAI viruses. In one study in Thailand of infection in ducks reared using different management systems, there were no reports of disease or infection in ducks reared intensively indoors despite sampling of all batches of ducks prior to market (60 samples for virus detection per flock). By contrast, for ducks reared in open sheds, four of 17 tested flocks were infected, as were 28 of 61 grazing flocks (Songserm *et al*, 2006).

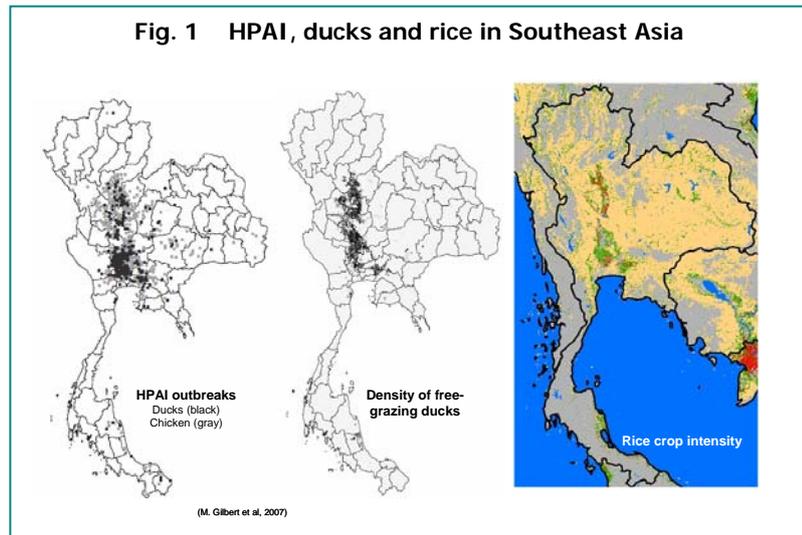
In intensive (so called 'X-ray') surveillance of village flocks in Thailand conducted in October 2004, 47 percent of backyard duck flocks were found to be 'infected' and were culled (Songserm *et al*, 2006). This demonstrated the high level of exposure of these ducks to infection at that time and suggests they played a significant role in the maintenance and spread of H5N1 HPAI viruses.

In Thailand, levels of infection in ducks appear to have been reduced through restrictions on grazing and culling of infected flocks (Tiensin *et al*, 2007a). These measures correlated with a reduction in cases in other poultry suggesting, but not proving, that ducks were playing an important role in the transmission of these viruses to other species.

### 3.1.2 Geospatial match between disease and ducks

An apparent association between ducks and outbreaks of disease has also been demonstrated through geospatial mapping studies (Fig 1).

Several studies have demonstrated an association between areas where ducks are allowed to graze (i.e. areas where rice cropping is practised and especially those areas with multiple rice crops every year) and the occurrence of H5N1 HPAI (e.g. Gilbert *et al*, 2006a).



These studies do not prove that domestic ducks are the only factor involved in the spread of the disease, but suggest a strong association. Other confounding factors associated with wetland agriculture could also be involved, notably wild birds which share the same ecosystem and, potentially, could be short-term subclinical or mechanical carriers of virus.

A similar occurrence is found in Viet Nam where most of the domestic ducks are located in the Mekong River delta and the Red River delta – the areas most affected by H5N1 HPAI. Here, attempts have been made to reduce the levels of infection in ducks through a combination of vaccination and restrictions on breeding and marketing, although recent outbreaks in ducks (in late 2006 and throughout 2007), especially in unvaccinated ducks, indicate that there is still scope to improve application of these preventive measures.

In China, the majority of ducks are reared in the south of the country, which is purported to be the 'epicentre' for emergence of many avian influenza viruses (Webster and Govorkova, 2006). However, the poultry subsector in the south of China also differs from that in the north with higher numbers of local 'yellow breeds' of chicken, and probably more sales of poultry through live-poultry markets (Sims *et al*. 2005). Thus, it is possible that these other factors also contributed to the higher levels of infection with H5N1 HPAI viruses that have been recorded in this region.

The information presented above suggests but does not prove a causal link between rearing of ducks outdoors and spread of H5N1 HPAI. However, when combined with experimental and field data showing that individual ducks can be infected 'silently' and excrete low levels of virus for a short period of time (usually about 1-2 weeks), it suggests that domestic ducks have played and perhaps continue to play a crucial role in persistence and spread of infection.

### 3.1.3 Mixing of ducks and other species

Mixed poultry farming is widespread across Asia especially in the village agricultural sector but also extends to some commercial flocks. Segregation of terrestrial and aquatic poultry is not practised on many small farms, during transport to market or in some live-poultry markets.

To assist in preventing the spread of HPAI in Hong Kong SAR, a complete segregation policy was imposed in 1998 for domestic waterfowl (Sims *et al*, 2003) and today it is still illegal to rear, transport or market ducks and geese together with other poultry. The value of this segregation was demonstrated between 1999 and 2000 when infection was detected in clinically normal geese and ducks sent from Guangdong province of China for slaughter in Hong Kong SAR but no infection was detected in terrestrial poultry that were marketed and transported separately during the same period. It was not until February 2001 that infection recurred in terrestrial poultry in markets in Hong Kong SAR.

Hence, it is likely that control of H5N1 HPAI in Asia and elsewhere would be facilitated by the separation of domestic waterfowl from terrestrial chickens, at least in markets and commercial farms. The difficulty of achieving this at the village level may prevent implementation in small flocks of scavenging poultry; alternative, practical means of control and prevention of infection may need to be explored in these situations.

### 3.1.4 Ducks and wild birds

Of all the types of poultry reared, domestic ducks are those most likely to have direct and indirect interactions with wild birds, given their shared habitat in wetlands/paddy fields. This provides opportunities for an exchange of viruses (in both directions) between these two populations. Water birds are not the only wild birds that can contact ducks in paddy fields. In Thailand, for example, many other types of free-flying birds can be found on paddy fields and these could play a potential role in dissemination of virus if they forage in areas frequented by infected ducks, even if just mechanical or short-term carriers of virus over relatively short distances. Recent experimental studies with Asian-lineage H5N1 HPAI viruses have shown that starlings (*Sturnus vulgaris*) and sparrows (*Passer domesticus*) can be infected for a short period with these viruses and that not all infected starlings die (Boon *et al*, 2007). It is not known whether these birds can carry or excrete sufficient quantities of virus to infect poultry, especially since some species of birds, such as wood ducks and turkeys, appear to require a lower infectious dose than chickens (Brown *et al*, 2007a, I. Brown, personal communication).

### 3.1.5 Ducks and water

Infected ducks transfer H5N1 HPAI viruses to the ponds, fields or wetlands they inhabit. Further, these viruses can survive in such environments for a variable length of time determined largely by temperature (three days in paddy field water at 25-32°C [Songserm *et al*, 2006] and longer under cooler conditions [Brown *et al*, 2007b]). The salinity of the water and possibly the levels of exposure to UV radiation may also play a role (Brown *et al*, 2007b, Stallknecht *et al*, 1990a). Asian-lineage H5N1 HPAI viruses appear to be less well adapted to aquatic conditions than LPAI viruses isolated from aquatic birds (Brown *et al*, 2007b).

Domestic ducks derived originally from Mallards (e.g. Pekin ducks) are the main species reared commercially around the world and are referred to as "dabblers". Dabblers tend to feed superficially (skimming the surface of water for feed), but can also feed on and filter mud in shallow waterways. This means that virus particles would have to be either suspended in water or deposited in mud in shallow water for these domestic ducks to become infected by this route. Ducks in water are also reputed to practise 'cloacal sipping' (in which water is sucked into the

cloaca), which could potentially enhance spread of infection if water is contaminated. However, no studies have been conducted on the dispersal of H5N1 virus particles deposited in pond or lake water. The rate of survival of virus in the littoral zone of lakes in places where infection is known to occur is an important area for future study.

The fate of respiratory-borne virus from ducks in water is not known. Since ducks are gregarious animals, the shift towards increased excretion of H5N1 virus via the respiratory route could potentially facilitate duck-to-duck transmission when birds are in close contact. However, studies of rates of transmission between ducks of viruses excreted predominantly via the cloacal or tracheal route have not been conducted.

### 3.1.6 Ducks and vaccination

Under laboratory conditions, properly formulated vaccines have been found to be successful at reducing and even preventing the shedding of H5N1 viruses in inoculated immune ducks subsequently challenged with virus (see for example Middleton *et al*, 2007; Beato *et al*, 2007). Given the many factors that complicate responses to vaccination, it is unlikely that the use of vaccines in the field will lead to elimination of virus, but experimental studies have shown that quantities of virus excreted can be markedly reduced such that transmission of H5N1 in a vaccinated flock would be far less efficient (and may not occur) than in flocks that remain unvaccinated. Vaccination is being used for containment of infection in ducks in China and Viet Nam but no controlled studies have been conducted to determine its overall efficacy under field conditions. The number of reported cases of disease in poultry and humans has fallen since widespread vaccination was introduced in both countries, but it has not been proved that this improvement was due solely to vaccination. In Viet Nam, outbreaks of H5N1 HPAI in 2007 mainly affected unvaccinated ducks.

In discussions on vaccination of ducks, some concerns have been expressed that the use of vaccines will drive antigenic change in H5N1 HPAI viruses. This is possible, but antigenic changes will also be driven by subclinical systemic infection in ducks, which also occurs when vaccination is not used. Selection of variant viruses has been demonstrated in experimental studies in unvaccinated ducks (Hulse-Post *et al*, 2005).

### 3.1.7 Duck eggs and day-old ducklings

No studies have been conducted on the presence of Asian-lineage H5N1 HPAI viruses in eggs from infected ducks but, based on experiences with other species, eggs laid by infected ducks could potentially contain some virus (Promkuntod *et al*, 2006). The surface of duck eggs is frequently soiled with faeces and since ducks can be subclinically infected and pass virus in faeces, contaminated eggs pose a potential transmission risk.

One investigation of cases of disease associated with H5N1 HPAI virus in young ducks in Viet Nam in 2007 suggested an association with specific hatcheries, but this was never demonstrated (see 3.3.2) (D. Hadrill, personal communication). Small-scale duck hatcheries in Viet Nam and elsewhere have the potential to transfer virus to newly-hatched ducklings through the use of straw and recycled transport containers (Sims, unpublished). Trade in these ducklings often involves several 'middlemen', who accumulate ducks from multiple sources, increasing the scope for contamination.

No studies have been published on the effect of maternal antibody to H5 avian influenza viruses on infection and virus excretion in day-old ducklings subsequently exposed to an H5N1 HPAI virus, and therefore the extent to which exposed ducklings with maternal antibody carry and excrete virus is not known.

### 3.2 GEESE AND H5N1 HPAI

Domestic geese (*Anser domesticus*) are believed to have played a key role in the evolution of Asian-lineage H5N1 HPAI viruses from the time they were first detected in 1996 and the emergence of multiple genotypes from 2000 onwards.

Geese were first found to be infected with the H5N1 HPAI virus in 1996 and again in 1997, and viruses similar to those found in 1996 were still circulating in geese from southern China in 1999 (Cauthen *et al*, 2000). By 2001, the original goose virus genotype was rarely encountered and was thought to have been replaced by a range of reassortants that often only retained HA and NA genes linked back to the original viruses (Guan *et al*, 2002b). Healthy geese were also found to be infected in Viet Nam in 2001 during market studies (Nguyen *et al*, 2005). These viruses differed somewhat from those found in 2003 and later.

Numerically, there are fewer geese (around 270 million) reared in Asia than ducks (about 930 million) and chickens (about 8.78 billion) (FAO, 2006), suggesting that they probably play a lesser role in the spread of infection than other infected species. Nevertheless, China still has a standing population of more than 268 million head of geese or 89 percent of the global goose population, which still represents a significant potential reservoir (FAO, 2006).

Because geese vaccinated with killed H5N1 vaccines do not appear to produce prolonged, high-level immunity under experimental conditions without the use of multiple, high doses of vaccine (Tian *et al*, 2005), these birds have the potential to play a role in virus persistence and spread if vaccination is used irregularly, especially if doses higher than those recommended for chickens are not used.

### 3.3 CHICKENS AND H5N1 HPAI

HPAI caused by Asian-lineage H5N1 viruses is one of the few diseases that is almost uniformly fatal for a particular species. In the case of chickens (*Gallus domesticus*) infected experimentally with these viruses by the intranasal or intravenous route, virtually all die rapidly (see for example Guan *et al*, 2002b). However, most experimental studies have used genetically-'improved' commercial chickens, with a few exceptions (notably some conducted in Hong Kong SAR which used local commercial Chinese breeds or cross-bred chickens).

There are a few anecdotal reports of chickens surviving infection in the field, although it is not clear whether these surviving birds were infected or merely exposed to the virus without subsequent development of infection. There are also some reports of small numbers of seropositive chickens in unvaccinated flocks. In one study from Viet Nam conducted one month after the last reported cases in the first wave of outbreaks in 2004 (and before introduction of vaccination), six percent of 379 chickens tested had antibodies to H5 virus, suggesting they had been exposed to an H5 virus and survived (Phuong *et al*, 2006). The possibility that this seroconversion was due to exposure to a low pathogenicity virus of the H5 subtype cannot be ruled out.

Chicken flocks probably do not remain infected with Asian-lineage H5N1 HPAI viruses for an extended period of time. In a commercial flock in which unvaccinated poultry are intensively reared, field experience suggests the virus will usually spread rapidly through the flock, especially if poultry are reared on litter at high stocking rates. In such cases, the virus will kill most poultry in the flock if the infection is allowed to run its course. This effect can be modified by the housing system. Observations in Hong Kong SAR during outbreaks in 2002 indicated that spread of disease in caged poultry was generally slower than for birds reared on litter (Sims, unpublished). One recent study using an H5N1 virus isolated from Japan in 2004 (Tsukamoto *et al*, 2007)

compared the rates of transmission under experimental conditions and found that spread was enhanced if more birds were infected initially and that spread by contact was more efficient than aerosol spread. This supported the view that housing systems and stocking density influence the rate of spread. Surprisingly, a recent analysis and model of cases of HPAI caused by H7N7 viruses in the Netherlands did not find a significant difference between housing systems and rate of spread, contrary to expectations (Tiensin *et al*, 2007b)

### 3.3.1 Day-old chicks

Commercially produced day-old chicks are unlikely to be infected with H5N1 viruses when they leave the incubator given the limited likelihood that eggs from infected hens would hatch and the poor prospects for survival of exposed virus at 37°C in incubators. However, no experimental studies have been conducted using eggs contaminated or infected with H5N1 HPAI viruses to prove this and therefore this possibility cannot be ruled out (Brugh and Johnson, 1986).

This suggests that if day-old chicks are spreading disease this is more likely to be via contact with contaminated transport containers or through exposure to infection after hatching. This could be facilitated by management practices such as the sale of day-old chicks in live-poultry markets, the use of natural brooding (especially the use of surrogate poultry to brood and hatch eggs) and, as with day-old ducklings, the use of recycled transport containers or contaminated straw for packing and movement of chicks.

A report from Viet Nam (Delquiny *et al*, 2004) argued that introduction of two day-old broiler chicks may have been responsible for the introduction of virus to a village in which detailed investigations were carried out. It was not possible to determine whether this was due to the chicks or fomite transfer. Other articles describing the situation in 2006 in Nigeria (Brown, 2006) and in India (Grain, 2006) suggested that day-old chicks may have been responsible for introduction of infection but these claims were never verified by formal studies. If similar cases occur in the future these should be investigated thoroughly to assess whether these were the result of infected chicks and, if so, how they were infected.

Outbreaks of H5N1 HPAI on multi-age farms in Hong Kong SAR in 2002 appeared to spare young poultry (Sims *et al*, 2003). Although the reason for this was not established, in some parts of Asia day-old chicks would have maternal antibody because they are derived from vaccinated breeders, and this may have afforded some protection from infection or disease for the first few weeks of life.

### 3.3.2 Chicken eggs and fomites

Some outbreaks of avian influenza have been associated with transfer of virus by egg flats (Cardona, 2005; Power, 2005). Chicken eggs from infected hens can potentially contain virus but, because the clinical course of HPAI in chickens is extremely short, it is unlikely that many infected eggs enter market chains. A theoretical risk remains but there are no reported cases of human disease associated with consumption or handling of eggs. Surface contamination of eggs is also a possibility both for HPAI and LPAI unless eggs are cleaned or disinfected. Thus infected and at-risk countries need to implement measures to prevent eggs or contaminated egg flats from spreading virus. This requires regular disinfection of egg flats whenever they are returned to a farm.

### 3.3.3 Native chickens, commercial chickens and resistance to infection

It has been suggested that native chickens may be less susceptible to infection with H5N1 HPAI (and other) viruses (Grain, 2006) but so far no experimental studies supporting this suggestion have been conducted, and evidence from the field remains anecdotal.

Considerable natural variation in the resistance of poultry to diseases exists and the mechanisms involved have been reviewed elsewhere (Zekarias *et al*, 2002). It has also been proposed that poultry resistant to HPAI could be bred, possibly through the introduction of RNAi transgenes to breeding stock (O'Neill, 2007).

It is possible that advantages conferred through natural selection make native poultry more capable of surviving under harsh village conditions (Kitaliyi, 1998) but there is limited evidence so far to suggest that this has led to selection of chickens resistant to HPAI. The only published data available are provided in a study of dead and live poultry from infected flocks in Thailand in which certain MHC class 1 haplotypes appeared to be associated with survival. (Boonyanuwat *et al*, 2006). This work needs to be verified through laboratory-based experiments.

If resistance to H5N1 HPAI is present in native chickens it is unlikely that this is due to selection pressure arising from infection with H5N1 HPAI viruses. HPAI has been recorded rarely in the past and there are no reports of H5N1 HPAI in Asia before 1996. It may, however, occur through selection for innate resistance to other viral infections such as Newcastle disease that have caused severe disease in poultry for many years.

No systematic studies have been conducted to assess whether absence of disease in certain village flocks or parts of flocks in infected areas is due to resistance to infection/disease or to low stocking densities, which could result in some poultry avoiding exposure to virus. Even commercial chickens are resistant to infection if exposed to very low doses of virus below the infective dose (Swayne, 2006) and some poultry in infected flocks can escape infection when stocking densities are low.

Under experimental conditions, virtually all chickens exposed to an infectious dose of Asian-lineage H5N1 HPAI die. This includes 'local' Chinese breeds (or their crosses) of chicken such as those raised in Hong Kong SAR, as demonstrated by experimental infection and by the high level mortality in some naturally-infected commercial flocks of these birds (Sims *et al*, 2003; Ellis *et al*, 2006).

Short-lived broiler chickens have been bred for growth characteristics which appear to have reduced and modified both their humoral and cellular immune responses compared with slower growing layers. (Koenen *et al*, 2002). This may affect their response to avian influenza vaccination and subsequent resistance to infection. Anecdotal reports suggest that serological responses in fast growing broilers vaccinated with vaccines containing killed H5 antigen are inferior to those produced in layers but no formal studies have been published on this. Such a difference, if demonstrated, could influence control and preventive strategies for H5N1 HPAI in broiler flocks.

It has been suggested that the gene pool of native breeds is more diverse than that of western-style broilers and this potentially provides greater protection against disease. Some studies have shown that genetic diversity was low in inbred poultry such as certain fancy breeds but higher in non-commercial Asian populations, which correlated with the management of the population studied (Hillel *et al*, 2003; Granevitze *et al*, 2007). However, this does not provide a conclusive answer to the question of whether certain local breeds and varieties of chicken are more

resistant to HPAI. This could also be confounded by the environment in which the poultry are reared, which will influence the level of viral challenge.

A number of recent articles have made the erroneous suggestion that village chickens live harmoniously with pathogens (see for example Grain, 2006). This does not reflect the high mortality seen in this production sector, much of which, in the past, was usually attributed to Newcastle disease (Johnston, 1990; Kitaliyi, 1998).

Nevertheless, some poultry do demonstrate some resistance to certain diseases (FAO, 1999) and no efforts should be spared in retaining genetic diversity of poultry to ensure that the special characteristics of native poultry and local breeds remain available. As will be discussed in Chapter 6, methods should be found to protect valuable breeding animals when stamping out H5N1 HPAI, as was done in Hong Kong SAR in 1997, allowing conservation of rare Chinese breeds in several uninfected flocks that would otherwise have been culled (Sims, unpublished). In addition, further work should be conducted to determine whether certain breeds display any natural resistance to infection or disease caused by Asian-lineage H5N1 HPAI viruses, including assessment of the role of specific variants of proteins such as Mx (Ko *et al*, 2002; Balkissoon *et al*, 2007) and MHC genotypes.

### 3.4 OTHER POULTRY AND H5N1 HPAI

A wide range of other poultry is produced and sold in Asia and elsewhere. This includes quail, pheasants, guinea fowl, partridges, pigeons, muscovy ducks and turkeys. The precise role of these species in the evolution, persistence and transmission of Asian-lineage H5N1 HPAI viruses remains poorly understood because little research has been carried out. Many of these birds are raised in small numbers together with chickens, ducks and geese in smallholder and village flocks, and are also sold together with other poultry in live-poultry markets, providing opportunities for cross-species infection.

Quail (*Coturnix spp.*) have attracted considerable attention because the internal genes of the H5N1 HPAI viruses from Hong Kong in 1997 were similar to those found in an H9N2 virus in quail (Guan *et al*, 1999). This suggested a possible role for this species in the evolution of the Hong Kong/97 genotype HPAI virus. Quail were also among the first avian species in which it was recognised that greater quantities of influenza virus could be excreted via the respiratory tract than in faeces, in contrast to earlier findings with avian influenza viruses in aquatic avian species (Liu *et al*, 2003). Outbreaks of H5N1 HPAI have been reported in quail in several countries, including Indonesia and Viet Nam. Experimentally, quail are highly susceptible to infection with H5N1 HPAI viruses (Perkins and Swayne, 2001).

It is no longer allowed to sell quail in live-poultry markets in Hong Kong SAR due to concerns about their role as a potential source of other influenza viruses that could reassort with H5N1 viruses. Quail are also known to be able to support growth of swine influenza viruses (Makarova *et al*, 2003).

Experimentally infected domestic pheasants (*Phasianus colchinus*) have the capacity to excrete some low pathogenicity avian influenza viruses for an extended period of time (>45 days) even in the face of antibodies to the virus. This has been associated with selection of antigenic variants during the infection process (Humberd *et al*, 2007), but it is unlikely that this also applies to H5N1 HPAI viruses (experimental inoculation of pheasants with a Hong Kong/97 H5N1 HPAI virus strain resulted in the death of all inoculated birds in 2.5-4 days [Perkins and Swayne, 2001]).

Little information is available on Asian-lineage H5N1 HPAI viruses in domestic guinea fowl (*Numida meleagris*) and partridges (*Alectoris spp.*). Guinea fowl experimentally inoculated with a Hong Kong/97 HPAI virus all died within five days (range 2-5 days, mean 2.5 days) (Perkins and Swayne, 2001). Some dead partridges naturally infected with H5N1 HPAI viruses were detected in Hong Kong SAR in 1997 (Sims, unpublished). In one experimental study using a Hong Kong SAR H5N1 HPAI virus from 1997, not all inoculated partridges died and those that died lived longer (4-6.5 days, 70 percent mortality) than other gallinaceous species inoculated with the same strain of virus. No virus was detected in the surviving birds 14 days after inoculation suggesting that they were not long-term virus shedders (Perkins and Swayne, 2001).

Domestic pigeons (*Columba spp.*) were largely resistant to infection with an H5N1 HPAI virus isolated from Hong Kong in 1997 (Perkins and Swayne, 2002) but infection and disease have been recorded in these birds with more recent H5N1 HPAI viruses both naturally and experimentally. Nevertheless, pigeons appear to be more resistant to infection than many other avian species (Boon *et al*, 2007; Werner *et al*, 2007) with high experimental challenge only resulting in development of neurological signs in some 20 percent of experimentally infected birds in one study (Klopfeisch *et al*, 2006). One recent study in China concluded that pigeons were resistant to infection using five different H5N1 HPAI isolates (Liu *et al*, 2007).

Clinically- and subclinically-infected muscovy ducks (*Cairina moschata*) have been detected in a number of countries including Viet Nam. Experimental infection of young muscovy ducks led to the development of neurological signs (Steensels *et al*, 2007). Virus shedding was detected up to 19 days after infection. Because these birds are often used as surrogate hens for hatching eggs in village flocks, they could, if subclinically infected, pass virus onto chicks when they hatch.

Turkeys (*Meleagris gallopavo*) appear to be inordinately susceptible to infection with avian influenza viruses based on the apparent disproportionate number of cases of avian influenza (including H5N1 HPAI) in Europe involving turkey farms (see for example Capua *et al*, 2003). They do not survive for more than a few days after experimental infection (Perkins and Swayne, 2001). As with other poultry species, turkeys incubating H5N1 HPAI virus may not show clinical signs. In the early stages of an outbreak, this could potentially lead to the inadvertent dispatch to slaughter of poultry containing infected birds. This is supported by the detection in early 2007 of presumably recently infected asymptomatic poultry in barns adjacent to a clinically affected flock on a turkey farm in the United Kingdom (Defra, 2007). Turkeys are a minor species of poultry in Asia, and therefore probably played little or no role in the initial evolution and emergence of Asian-lineage H5N1 viruses.

### 3.5 WILD BIRDS AND H5N1 HPAI

Wild aquatic birds have been incriminated in a number of outbreaks of HPAI as the source of LPAI viruses that subsequently converted to highly pathogenic strains in poultry (see Table 2.1 and Swayne and Suarez, 2000). However until the emergence of Asian-lineage H5N1 HPAI viruses, wild birds were not regarded as a primary source of HPAI viruses.

Until 2002, only a few individual dead wild birds infected with HPAI viruses had been detected and only one outbreak of HPAI (H5N3) involving multiple wild common terns (*Sterna hirundo*) in South Africa in 1961 had been reported [Becker, 1961]). H5N1 HPAI has changed the way we view wild birds and HPAI – not only have numerous wild birds been killed by H5N1 HPAI viruses, but the evidence presented in Chapter 2 strongly suggests that they have played a role in the long-distance spread of these viruses.

As discussed in Chapter 2, cases in dead wild birds have been identified for which no association with poultry has been found, including a range of species in Europe and wild water birds in

Mongolia. Although the source of these viruses is not known and they may have been derived originally from poultry, it has been possible through a process of elimination to rule out poultry or items related to poultry as the direct route of introduction.

Infected dead 'wild' birds have also been found in Hong Kong SAR where domestic poultry in commercial farms have remained free from infection since 2003 (AFCD, 2006; AFCD, 2007). Some of these infected wild birds could have been part of shipments of captured birds imported largely for religious functions. The current evidence for this is circumstantial, based on the species affected and the locations where the dead birds were found (Young, 2007). Viruses isolated from dead free-flying birds in Hong Kong SAR in 2006 and 2007 differ genetically from those detected in poultry in 2002-03 (OIE, 2006b) indicating that these were not derived from earlier outbreaks in poultry. It is also extremely unlikely that these were derived from the few remaining small flocks of backyard poultry given the very low number of such birds in Hong Kong SAR.

Ornithologists have pointed out that the timing and pattern of disease outbreaks in poultry do not always coincide with known migratory patterns (Bird Life International, 2006). Although this is true, it does not reflect the fact that bird movements are not as precise as existing maps suggest (EFSA, 2006). This was demonstrated by the apparently aberrant movement of water birds in the northern winter of 2005-06, associated with freezing conditions around the Black Sea. Many birds are nomadic, moving in accordance with availability of feed and water, rather than long-distance migrants following strict patterns of movement (see for example Tracey *et al*, 2004; EFSA, 2006). This could possibly explain some of the movement of virus that has occurred but which does not fit with accepted 'normal' migratory patterns.

In addition, coincidence of infections in poultry with times of migration is not essential because amplification of virus in resident wild birds or domestic ducks could occur before virus spreads to domestic chickens and infection becomes apparent. Delays between introduction of infection by wild birds and infection in poultry has been demonstrated with LPAI viruses in farmed turkeys in Minnesota, United States, in the early 1980s where there was an apparent lag between introduction of the viruses by wild ducks and subsequent infection of the turkeys (Halvorson *et al*, 1985).

Despite intensive surveillance of wild birds, only a few 'healthy' wild birds have been found to be infected with H5N1 viruses – most have been found sick or dead. The infected live birds include at least one grebe from Siberia in 2005 (Lvov *et al*, 2006), healthy swans from Poland in 2006 (Minta *et al*, 2006) and a total of six out of 4 300 wild ducks found in Poyang Lake, China, in early 2005 (Chen *et al*, 2006c). It is worth noting that had the viruses from the Poyang Lake ducks (species not identified) reassorted they could have produced a virus similar to that found in dead and dying birds in Qinghai, China, in May 2005 (Chen *et al*, 2006c). In addition, healthy tree sparrows (*Passer montanus*) were found to be infected with a novel genotype of HPAI H5N1 virus in China in 2004 (Kou *et al*, 2005).

The difficulty in detecting virus-positive live birds could relate to the very low prevalence of healthy carriers. Based on experimental information involving a limited range of species, most birds infected with H5N1 HPAI viruses do not excrete virus for more than a few days, although some ducks will excrete virus for several weeks. It may also reflect sampling inadequacies given that current HPAI viruses are excreted at lower levels by the cloacal route than the respiratory route in experimentally-infected mallards, although in at least one duck in this study, cloacal excretion extended over a longer period (Hulse-Post *et al*, 2005).

It is also evident that wild birds can be infected by poultry, as was seen in large-billed crows (*Corvus macrorhynchos*) in Japan (2004) possibly scavenging on chickens that died from H5N1

infection (Nishiguchi *et al*, 2005) and most likely with hooded vultures (*Necrosyrtes monachus*) in Burkina Faso in 2006 (Ducatez *et al*, 2007). The infected crows in Japan were found for up to 30 days after the farm cases ceased. Most of these were probably infected after scavenging dead poultry but the possibility of a short-term independent cycle of infection in the crows cannot be ruled out.

Much remains unknown about the survival of H5N1 HPAI viruses in wild bird populations, including the important question of whether infection is sustained in these without replenishment from other sources, especially infected domestic poultry. Since 2005, only H5N1 viruses of subclade 2.2 have been detected in wild birds and poultry to the north and west of China, and in Japan and the Republic of Korea, despite the detection of a range of other clades and subclades in poultry in China and other parts of eastern Asia.

H5N1 HPAI viruses have been detected in many parts of the Palaearctic, so the persistence of virus in water over the northern winter in colder areas remains a possibility. This has been proposed as one of the methods of survival of other avian influenza viruses from year to year (Stallknecht, 1997), with the alternative being that viruses continue to circulate year round at low levels in wild bird (duck) populations. If the former occurs, healthy migratory birds returning to infected locations could pick up residual viruses that have survived over the cold months. Either of these infection pathways would hinder attempts at global eradication of Asian-lineage H5N1 HPAI viruses.

### 3.6 MAMMALS AND H5N1 HPAI

Cases of H5N1 HPAI have been recorded in cats (both domestic cats and captive wild felids) in areas where infection was present in poultry. Some onward transmission of virus has occurred among kittens in close contact experimentally, suggesting that virus could persist in a litter for at least a limited period (Rimmelzwaan *et al*, 2006). The role of cats in transmission of virus to humans or other species remains to be determined. However, as long as a cycle of infection does not become established in these animals, their role will remain secondary to that of infected poultry. The possibility of endemic infection in cats in Indonesia warrants further examination.

There is no evidence to suggest that H5N1 HPAI viruses are adapted to swine (Choi *et al*, 2005), although there have been isolated incidents in which pigs were found to be infected (Li *et al*, 2004b). At this stage, pigs should be monitored in areas where disease is occurring in poultry and ongoing serosurveillance of pigs at slaughter should be conducted to ensure that H5N1 viruses have not become established in this species.

As discussed in Chapter 2, H9N2 avian influenza viruses have been detected frequently in pigs (Peiris *et al*, 2001; Chen *et al*, 2006b) in China and theoretically pose a greater risk of spread to humans than H5N1 HPAI viruses given that they have already adapted to infect a mammalian host.

Cases of HPAI infection have been recorded in a number of other animal species, both naturally and experimentally, including laboratory mice and ferrets (Maines *et al*, 2005), Owston's civet (Robertson *et al*, 2006), stone marten (WHO, 2006) and dogs (Thiry *et al*, 2007). As with cats, these all remain potential vectors of spread, but unless independent cycles of infection develop in any of these species the control of the disease in poultry will also limit their importance in spread of Asian-lineage H5N1 HPAI viruses.

### 3.6.1 Transmission of Asian-lineage H5N1 avian influenza viruses to humans

The transmission of HPAI viruses from birds to humans is a serious public health concern and has been reviewed in depth elsewhere (see for example Peiris *et al*, 2007). Although Asian-lineage H5N1 avian influenza viruses pose a potentially serious threat of converting to a human pandemic strain, they are not yet easily transmissible to and between humans. They do not replicate efficiently in humans, although some subtypes of avian influenza can replicate within the human respiratory tract and cause disease. As of 14 December 2007, there had been 340 confirmed clinical cases of H5N1 influenza, of which 208 had been fatal (WHO, 2007). This probably underestimates the true number of cases but given the very large number of humans exposed to H5N1 HPAI viruses over the past 11 years, it is apparent that the attack rate for this disease is extremely low (i.e. many exposures but few cases of infection/disease). For example, studies in Nigeria suggest that despite considerable circulation of virus, no seroconversion in humans has been detected in high-risk occupational groups (Ortiz *et al* 2007), although there has been one confirmed fatal human case in that country.

Across Asia, most human cases have occurred in places where poultry are kept and where, usually, sick poultry are detected concurrently. Apart from a few instances where human-to-human transmission may have occurred (Kandun *et al*, 2006; Yang *et al*, 2007), most human cases have a probable link to infected poultry (Peiris *et al*, 2007).

Case control studies for human cases of HPAI have been performed in a number of locations, including Hong Kong SAR, Indonesia, Thailand and Viet Nam. The case control study in Thailand suggested that contact with inexplicably dead poultry was the strongest risk factor (Areechokchai *et al*, 2006). This differed from the situation in Hong Kong SAR in 1997 where a visit to a market selling live poultry was found to be a significant risk factor (Mounts *et al*, 1999), although it is now known that the live-poultry markets in Hong Kong were heavily infected at that time.

Much remains unknown about the factors that determine susceptibility to infection after exposure in humans. The possibility of a genetic determinant of susceptibility based on clusters in blood relatives has been suggested by some, although so far there is little evidence to support this (Pitzer *et al*, 2007). It is an area that warrants further exploration.

At present, the main role of humans in the spread of avian influenza is through activities related to the production and marketing of poultry. These are discussed in detail in Chapter 4.

If a human pandemic strain of influenza virus emerges (one capable of sustained human-to-human transmission) from an avian influenza virus, poultry will likely become largely irrelevant in the spread of the virus, which will occur instead through movement of infected people.

## 3.7 CONCLUSIONS

The evidence presented above strongly suggests that domestic ducks – birds that in most cases are reared extensively – have played a key role in the genesis of new genotypes and spread of Asian-lineage H5N1 HPAI viruses.

It is not clear exactly how infected ducks infect other poultry. However, there is considerable sharing of environments by ducks and other poultry, especially at village level and in some live-poultry markets. Water contaminated with viruses from ducks could also transmit the virus to other avian species.

As long as ducks are reared under management systems with poor biosecurity, including free grazing and channel and pond rearing, they represent a risk that H5N1 HPAI will be spread and

maintained. In areas where infection is present, methods will be required to protect these birds from infection and to stop them becoming a source of infection for other poultry and possibly wild birds.

Thailand has done this by restricting the grazing of ducks and culling known infected flocks. The effect of these measures cannot be isolated from those due to other measures, but overall there was a marked fall in the number of reported cases in 2006 compared with 2004. It is highly likely that measures related to ducks were a key factor in controlling HPAI there. However, care needs to be taken in changing management practices for ducks to ensure that the ecological balance is not changed given the role that ducks are likely to play in pest management and fertilization of rice paddies.

Other countries such as Viet Nam are promoting a shift away from grazing ducks to intensive duck production but this requires substantial investment and increased inputs for feeding and maintenance of duck flocks. Given the size of the duck population in Viet Nam, it seems unlikely that more than a small percentage of these will be reared indoors under intensive conditions for some years to come. Nevertheless, if reared under biosecure conditions, experiences from Thailand suggest that they can be kept free from infection.

Vaccination is the other method available for reducing the virus load in ducks and is being used in China, Indonesia and Viet Nam. Use of vaccine in ducks will likely be required for some time given that there are few other ways of significantly enhancing biosecurity for ducks reared outdoors on rice fields, ponds or channels.

Wild birds have almost certainly played some role in the spread of H5N1 HPAI viruses but as will be seen in Chapter 4 their relative contribution is likely to be lower than that associated with human activities involving rearing and marketing of poultry, especially in those countries where infection persists in poultry.

The main conclusion that can be drawn so far from human cases is that the majority of these occur in rural settings where there is close association with poultry. Humans have acted as sentinels for infection in poultry indicating that there is considerable under-reporting of this disease in poultry in rural areas. Cases where disease has not been closely associated with sick poultry have occurred in places where the virus is known or suspected to be present in live-poultry markets, or sufficient samples have not been tested to rule out this possibility. Spillover infections have occurred in other mammalian species.