Appendix Three: Bovine tuberculosis in buffaloes, Southern Africa

(Appendix reference: de Garine-Wichatitsky, M., Caron, A., Gomo, C., Foggin, C., Dutlow, K., Lane, E., Le Bel, S., Hofmeyr, M., Hlokwe, T. and Michel, A. 2010. Bovine tuberculosis in buffaloes, Southern Africa. *Emerging Infectious Diseases*, 16 (5) 884-885)

To the Editor: Emerging tuberculosis in Southern African wildlife has implications not only for the conservation of wildlife species affected (Caron et al. 2003), but also for the health of human and livestock living at the wildlife-livestock-human interface (Michel et al. 2006). First diagnosed in African buffaloes (*Syncerus caffer*) in South Africa's Kruger National Park (KNP) in 1990 (Bengis et al. 1996) , the disease was likely introduced in the park by cattle-to-buffalo transmission (Michel et al. 2009). Bovine tuberculosis (bTB) infection is spreading northward: in 2003 infection was confirmed in a buffalo approximately 60 km south of the Limpopo river; in 2005 a case was confirmed only 6 km south of the Limpopo (D. Keet, unpub data).

We report the first isolation of *Mycobacterium bovis* from African buffaloes in Zimbabwe. During a survey carried out 9th-13th October 2008, 38 buffaloes from four different herds were captured in Gonarezhou National Park (GNP; south of Mabalauta area; 22.0553°S; 31.4265°E). Blood samples collected from immobilised buffaloes were tested using a gamma interferon assay (Grobler et al. 2002). All sampled buffaloes were marked and released, and three adult females in each group equipped with radio-collars. Four buffaloes (9.5%) tested positive for bTB in the gamma interferon assay, including two adult females and one subadult male from the same herd, and one adult female from another herd. Four months later a collared adult female and the sub-adult male, which had both tested positive on gamma interferon assay, were traced and darted by helicopter. Following euthanasia both animals were necropsied in the field and samples collected from lymph nodes of the head and thorax for histopathology and culture. No acid-fast organisms were detected but the histological findings were strongly suggestive of paucibacillary tuberculosis. *M. bovis* was isolated from the retropharyngeal lymph nodes of both buffaloes and from the bronchial and head lymph nodes of one buffalo. Both isolates were typed by analysis of variable numbers of tandem repeat (VNTR) sequences using six loci (ETR A-F) (Frothingham and Meeker-

O'Connell 1998) and compared with the VNTR profiles of approximately 75 isolates from the KNP. All isolates showed an identical VNTR profile (7544*5 2.3), suggesting an epidemiological link between the *M. bovis* infections in the two parks. However, the ETR loci were shown to have a lower discriminatory power among KNP isolates than IS*6110* restriction fragment length polymorphism typing (Hlokwe, unpublished data) (Michel et al. 2009) and a typing regimen comprising different typing methods and markers will be useful to determine the genetic relationship between the isolates from Gonarezhou and KNP more accurately.

The confirmation of bTB infected buffaloes in Zimbabwe GNP raises a number of questions regarding the spread of transboundary animal disease and has considerable management implications for the Great Limpopo Transfrontier Conservation Area (GLTFCA). The most likely scenario is buffalo-to-buffalo contact across the boundary, because the bTB cases reported here were located less than 45 km from the (unfenced) northern boundary of KNP. Buffaloes frequently disperse between herds, especially bulls and young heifers, and may contribute to the spread of *M. bovis* by mixing with naive herds (Cross et al. 2005). Although transboundary movements of buffaloes between KNP and GNP have not been specifically documented, uncontrolled movements across the Limpopo do occur (de Garine-Wichatitsky, personal observation). However, more than 12 wild species have now been demonstrated to be infected by bTB in the KNP (Michel et al. 2006). Most of them are probably not an effective source of *M. bovis* for buffaloes, but bTB epidemiology could rely on multi-host reservoir (Renwick et al. 2007). Thus, a second scenario could involve a buffalo-to-unidentified wild species-to buffalo pathway, as species like greater kudu (*Tragelaphus strepsiceros*) appear to be able to maintain, spread and even drive a bTB epidemic in some cases (Keet et al. 2001, Michel et al. 2009). The third scenario involves movements of infected livestock across the boundaries of the three countries of the GLTFCA,

via a cattle-to-buffalo contamination. As a last scenario, we cannot rule out the possibility that bTB infection of buffaloes has remained silent and undetected for decades in Zimbabwe.

The management implications of the discovery of bTB in buffaloes from GNP are considerable. Once established in a native free-ranging maintenance host, eradication of bTB is unlikely (*2,10*), and there is an urgent need to evaluate the prevalence and the distribution of the infection in wildlife and livestock populations on the Zimbabwean side of the GLTFCA. Control options of bTB in wildlife are limited (De Lisle et al. 2002, Michel et al. 2006), but chances of success are greater if control measures are initiated at the early stage of the disease spread into a new area. Adequate risk mitigation strategies should also be developed and implemented to reduce the risk of bTB transmission to livestock and humans living at the periphery of the unfenced GNP. Failure to promptly assess the situation and adopt appropriate measures would have far reaching conservation, economic and public health consequences, not only for Zimbabwe, but also for the political and social acceptance of the TFCAs that have been blooming in Southern Africa.

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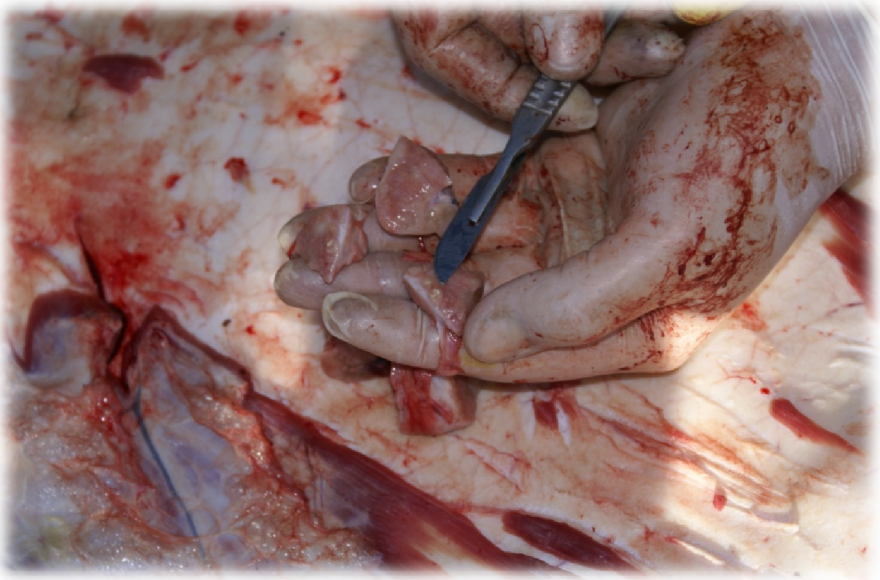


Appendix Four: Understanding the ecological drivers of avian influenza virus infection in wildfowl: a continental scale study across Africa

(Appendix reference: Gaidet N., Caron A., Cappelle J., Cumming G.S., Balança G., Hammoumi S., Cattoli G., Abolnik C., Serva de Almeida R., Fereidouni S.R., Grosbois V., Tran A., Mundava J., Fofana B., ould Elmamy B., Ndlovu M., Mondain-Monval J.Y., Triplet P., Hagemeijer W., Karesh, W. B., Newman S.H., Dodman T. 2011. Understanding the ecological drivers of avian influenza virus infection in wildfowl: a continental scale study across Africa. In press in *Proceedings of the Royal Societies Series B*)

Introduction

Infectious zoonoses are a growing concern as the human population expands and contact rates between humans and animals increase (Daszak et al. 2001). A majority of zoonoses are caused by pathogens with a wildlife origin. As we seek to understand and control zoonoses, the influence of host ecology on pathogen transmission is increasingly being recognised as fundamental to the dynamics of wildlife zoonotic disease (Alitzer et al. 2006, Stallknecht 2007, Carver et al. 2009, Tompkins et al. 2010).

Although considerable progress has been made in recent years in linking ecological and epidemiological perspectives, empirical explorations of the interface between ecology and epidemiology are still in an earlier phase (Carver et al. 2009, Tompkins et al. 2010). We currently lack a widely accepted theoretical framework that captures both the ecological determinants of disease transmission and classical epidemiological dynamics. One of the most obvious current barriers to the development of such a framework is the absence of data-heavy empirical tests of hypothesized mechanisms (Carver et al. 2009, Tompkins et al. 2010). Detailed empirical evidence is needed from host population to community levels and across a variety of environmental conditions, scales, and host communities. We address this need through a detailed, continental-scale, empirical analysis of some of the ecological mechanisms underpinning the transmission and perpetuation of avian influenza viruses (AIV) in their main natural reservoir, wildfowl (ducks, swans and geese).

AIV offers an informative case study because it occurs globally with high variations in prevalence in a range of highly mobile and abundant host populations (Olsen et al. 2006), creating both the potential to explore a wide range of environmental influences on transmission and the potential to better understand the role of animal movement and seasonal fluctuations in animal abundance in disease dynamics. The ecology and epidemiology of AIV

have received significantly increased attention in recent years in response to the emergence and spread of the highly pathogenic avian influenza (HPAI) H5N1 viruses across Eurasia and Africa (Hoye et al. 2010), responsible for human fatalities and large economic losses in the poultry industry. Several mechanisms have been proposed whereby host ecology and the environment may influence the dynamics of AIV transmission in wild bird populations (Webster et al. 1992, Krauss et al. 2004, Olsen et al. 2006, Munster et al. 2007, Stallknecht 2007, Munster and Fouchier 2009). However, few empirical investigations of these mechanisms have been conducted in particular across large spatial scales (Hoye et al. 2010).

In what follows, we first present the general ecological factors operating at the host population and community levels and through seasonal environmental drivers, and then provide a detailed analysis of their potential influence on the prevalence of AIV infection measured in a large-scale study of AIV in wildfowl (Figure A4.1). Table A4.1 summarises current understanding of AIV transmission mechanisms in wildfowl.

Although this summary captures many of the basic elements of a general model that links epidemiology and ecology in the context of infectious zoonoses, it is important to note that most of our understanding of AIV infection dynamics is derived from studies that have been conducted in boreal or temperate regions of the northern hemisphere (Krauss et al. 2004, Olsen et al. 2006, Munster et al. 2007). There is a knowledge gap in tropical regions, and in particular in sub-Saharan Africa. Earlier studies have suggested that tropical regions may act as an epicentre contributing to year-round AIV perpetuation in wild birds (Webster et al. 1992). More recently, AIV have been found circulating in various regions of the African continent in both Afro-tropical and migratory Eurasian wildfowl (Appendix Two - Gaidet et al. 2007, Abolnik et al. 2010, Chapter Four - Caron et al. 2011) indicating that local environmental conditions are favourable for the transmission of AIV. The patterns of AIV prevalence observed in temperate or boreal regions cannot be directly transposed to the

tropics where differences in host ecology, climatic constraints and seasonality may produce different dynamics of infection.

In Afro-tropical regions, seasons are determined by rainfall rather than temperatures. The annual migration of the inter-tropical convergence zone (ITCZ) produces a distinct wet season of variable duration according to latitude (Figure A4.1A). The Afro-tropical regions are characterised by high temperatures of relatively low annual variation. During the rainy season many Afro-tropical wetlands experience extreme seasonal variations in their surface area (Conway et al. 2009). Rivers may swell rapidly, after a relatively short but intense rainy season, with the capacity to inundate vast floodplains. Many wetlands are ephemeral, due to the long dry season and high evaporation rates. At the end of the dry season, water bodies are generally limited to a few permanent wetlands where waterbirds congregate.

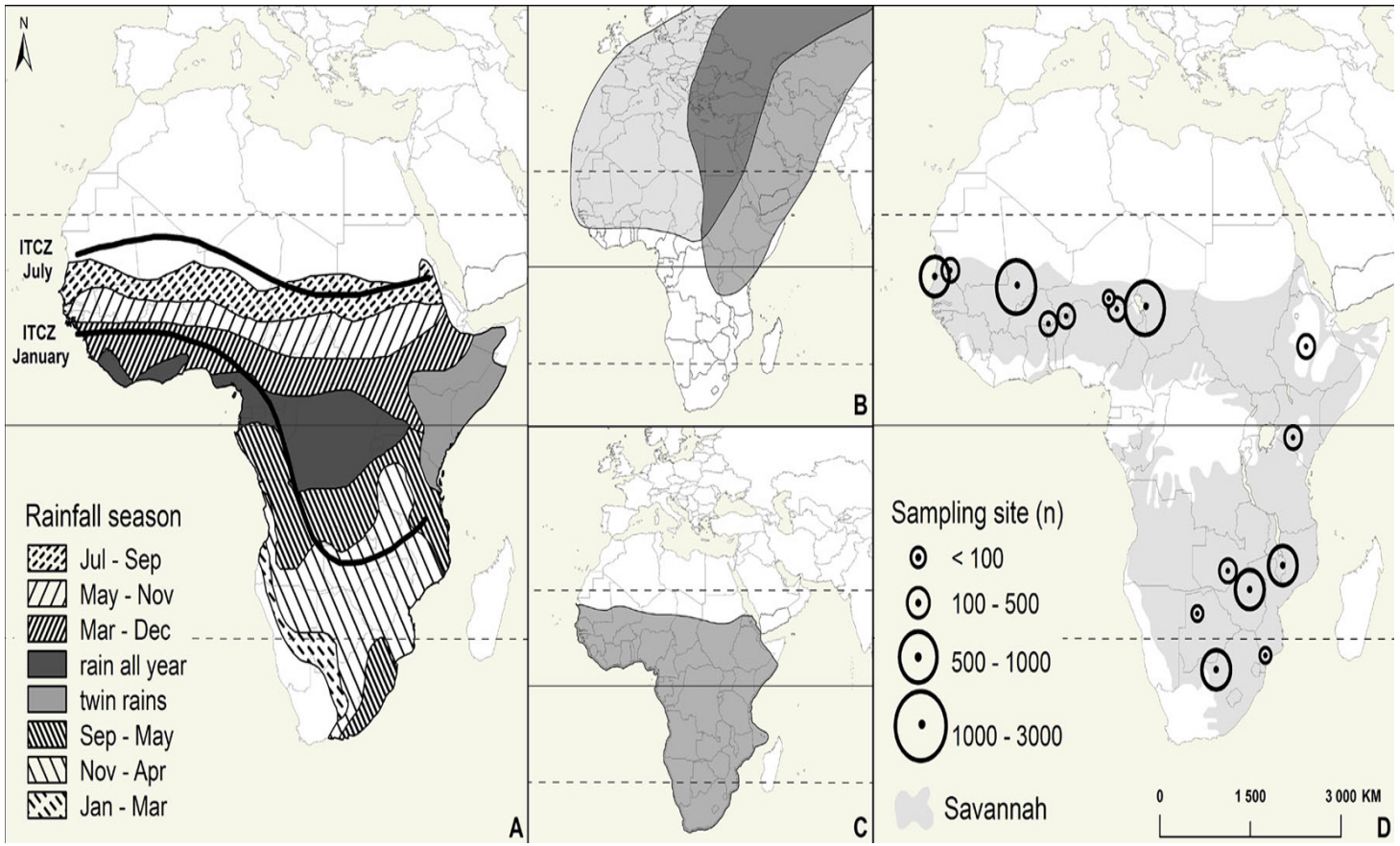
Sub-Saharan Africa north of the equator constitutes a seasonal non-breeding area for a large number of Eurasian (i.e. Palaearctic-breeding) migratory ducks between September and March (Zwarts et al. 2009) (Figure A4.1B), including eight widespread species and four uncommon species (Wetlands International 2006). Most are dabbling ducks of the *Anas* genus, the most numerous being Garganey (*A. querquedula*) with populations of c. 2 million birds (Scott and Rose 1996). All African regions, including those south of the equator, are also connected to Eurasia by other Eurasian migratory waterbirds including waders, gulls, terns, rails, herons and storks.

***Table A4.1**: Summary of our current understanding of AIV transmission in wildfowl in relation to host ecology and environmental drivers.*

Epidemiological parameters	**Experimental[1] or empirical[2] evidence and epidemiological predictions[3]**	**Sources of ecological variations**	**Ref.**
Environmental transmission	[1]AIV can remain infectious for several months in water under experimental conditions. Warmer temperatures, radiation and desiccation reduce the duration of AIV infectivity	Climatic constraints on the persistence of AIV in the environment	8,12
	[2]Mathematical water-borne transmission models captured some patterns of AIV infection dynamics in wildfowl		13,14
	[1]Successful experimental infection of ducks by contact with contaminated water	Species foraging behaviour affecting host exposure to environmental infection	15
	[2]Higher prevalence in dabbling ducks (foraging mainly in surface water) than in diving ducks (feeding in deeper water) or grazing wildfowl (foraging on grasslands)		6,11
Inter-individual transmission	[3]Density-dependent transmission may occur if the contact rate between susceptible and infected birds increases with host density	Host density and seasonal congregation affecting contact rate	16
	[2]The northern autumn peak in AIV prevalence in ducks coincides with bird seasonal flocking at pre-migration and stopover staging are		8,9,17
Transmissibility	[3]Potential intrinsic differences in permissiveness to AIV infection between wildfowl species accounted by differences in distribution of virus receptor types	Species evolutionary history	8,11
	[2]Higher prevalence consistently reported in *Anas* compared to other wildfowl species		6,10
Host susceptibility	[3]Immunological naivety of birds with no previous exposure to AIV increase their susceptibility to infection	Host age and species social or migratory behaviour influence on previous AIV exposure	8,11,18
	[1]Evidence of cross-protective immunity against AIV re-infection		19
	[1]Age at infection affects the extent of viral shedding in experimentally infected wildfowl		20
	[2]A higher AIV prevalence in hatch-year birds compared to after-hatch-year birds is consistently reported		10, 21
Herd immunity	[3]The proportion of susceptible individuals in the host population may control disease transmission rate	Demographic rate and migration influx affecting turnover of susceptible hosts	2
	[2]Proportion of hatch-year bird in wildfowl populations gradually decreased along the flyway during the autumn postnuptial migration		22
	[2]Prevalence decline during Northern autumn and winter as the proportion of naïve birds progressively decreases through infection or death	Timing of reproduction and congregation influence seasonal fluctuation in population immunity	9,23, 10,17
Host dispersal	[1]Experimentally infected wildfowl generally excrete a high concentration of AIV for 1-3 weeks without apparent signs of disease	Range and timing of host migration depending on species	19,20

	[2]Migratory wildfowl are able to perform long-distance movements within the time frame of AIV infection	and breeding regions	24
	[2]Phylogenetic analysis confirms the occurrence of inter-continental exchange of viruses via migratory wildfowl		25
	[2]Phylogeographic analysis suggest a dominance of migration over persistence process in the interannual perpetuation of AIV in wildfowl		26

Figure A4.1: *A. The timing and duration of rainfall in sub-Saharan Africa and the seasonal position of the Inter-Tropical Convergence Zone (ITCZ) (30); B. The two main migratory flyways of Eurasian wildfowl wintering in sub-Saharan Africa; C. Distribution range of Afro-tropical wildfowl over the African continent; D. Location of sampling sites presented in our study, also showing sample size ranges.*



In their wintering sites, Eurasian ducks congregate and mix with Afro-tropical wildfowl that reside year-round within sub-Saharan Africa (Figure A4.1C). The diversity of Afro-tropical wildfowl (31 species) is similar to that of Eurasian wildfowl (39 species), but only a few species (n=3) have a population in excess of 500,000 birds (Wetlands International 2006). Several Afro-tropical wildfowl species are widespread over sub-Saharan Africa, some of which are at least partially migratory, including trans-equatorial movements (Scott and Rose 1996). Breeding generally occurs during or following the wet season, but Afro-tropical wildfowl have extended breeding seasons compared to Eurasian wildfowl, with laying periods stretching over 6 to 12 months (Brown et al. 1982).

Given the many differences between temperate and tropical environments, it is unclear how well the elements of a general framework presented in Table A4.1 capture key dynamics in Afro-tropical ecosystems. To address this question, and to extend our empirical knowledge of AIV, we undertook a large-scale analysis of data on AIV in free-living wildfowl that were collected from 15 African countries (Figure A4.1D, Supplementary Information (SI) Table A4.S1) during 2006-2009. We analysed host, seasonal and geographical variations in AIV prevalence and focused on two potential but non-exclusive processes that potentially control the dynamics of AIV transmission in Afro-tropical regions: a seasonal introduction and spillover of AIV from Eurasian migratory waterbirds, and an endemic cycle (i.e. a year-round perpetuation of AIV by wildfowl in Afro-tropical ecosystems).

Our analysis is structured around six predictions. If Eurasian migratory waterbirds, and in particular Eurasian wildfowl, are the prime source of introduction of AIV in Afro-tropical regions, we predict that local and seasonal AIV prevalence should be related to: i) the geographic origin of species sampled, with a higher prevalence in Eurasian than in Afro-tropical wildfowl; ii) the proportion of Eurasian wildfowl species in the local wildfowl community; and iii) the timing of arrival of Eurasian migratory waterbirds, with a seasonal

peak in prevalence when migrants arrive followed by a decrease as the level of population immunity increases.

In addition, in the case of an endemic AIV cycle in Afro-tropical regions, we predicted iv) a higher prevalence in *Anas* than non-*Anas* species irrespective of their geographic origin. We also expected transmission of AIV to be driven by ecological factors homologous to those proposed for temperate and boreal regions (Webster et al. 1992, Krauss et al. 2004, Olsen et al. 2006, Munster et al. 2007, Munster et al. 2009) but adapted to the ecological context of Afro-tropical ecosystems. If climatic constraints limit the survival of AIV in the environment in the tropics, we predicted that v) AIV prevalence would be influenced by ecological factors associated with density-dependent transmission (host density and seasonal congregation) rather than with environmental transmission (climatic indices and species foraging behaviour). Lastly, since Afro-tropical regions are characterised by a more gradual rate of recruitment of susceptible juveniles (due to extended breeding seasons), and since seasonal congregation of wildfowl is related to the progressive reduction in the surface of wetlands during the dry season rather than to flocking before and during migration, we predicted that vi) the seasonality of AIV transmission will increase progressively from the onset of the wet season to the end of the dry season in response to both a progressive supply of susceptible hosts and a progressive congregation of wildfowl at permanent water bodies during the dry season.

Materials and Methods

Samples originated from apparently healthy live-caught birds captured using mist-nets and baited walk-in traps, or from freshly killed birds provided by hunters. Samples were collected throughout the year (with at least two months between sampling occasions). Birds

were tested for AIV from three types of samples that we distinguished in our analyses: double (cloacal + oropharyngeal swabs tested individually), single cloacal or single oropharyngeal. Samples were analysed in different laboratories using a similar standard diagnostic procedure based on RNA extraction and real-time RT-PCR virus detection (see SI Methods for a complete descriptions of sampling and diagnostic methods). We estimated prevalence for each species, sampling site and sampling occasion, as the percentage of individuals found positive for AIV as compared with the total number of birds tested.

Ground-based and satellite-based data were used to estimate explanatory variables used to quantify the ecological factors considered in our analysis (see SI Methods). We used a generalized linear modeling approach to relate ecological factors to the prevalence of AIV in wildfowl. A beta-binomial distribution with a logit link function (function betabin in the R-package aod) was applied to account for the aggregations of positive birds within our samples. We then used an information-theoretic procedure based on the Akaike information criterion corrected for small sample sizes (AIC_c) to select the most parsimonious model from among a set of candidate models with different combinations and numbers of variables (Burnham & Anderson 2002). We ranked models using ΔAIC_c, i.e. the difference of AIC_c values between a given model and the model with the lowest AIC_c values in the set of models. We also computed Akaike weight (ω_i) for each model *i* that gives the relative likelihood of a given model to be the best among a set of models fitted. We estimated the relative importance of each explanatory variable by summing the Akaike weights ($\Sigma\omega_i$) of all models in the set where that variable occurred. We tested for a quadratic relationship for variables whose influence was predicted to show a cyclical annual variation (time/ arrival Eurasian migrants and time/onset the wet season). Since inter-annual variations in AIV prevalence are commonly reported for wildfowl (Krauss et al. 2004, Munster et al. 2007), we controlled for

the potential confounding influence of individual years in our analyses, as well as for two other 'nuisance' parameters (laboratory and sample type).

Our approach consisted of three steps. First, we determined the core variables for each factor presenting alternative explanatory variables (Table A4.S4), testing successively each variable by permutation in a set of global models. Second, we evaluated the relative importance of each ecological factor by building a set of candidate models representing all possible linear combinations of the core variables, testing non-independent variables successively by permutation. Finally, we computed intercepts and model coefficients through model averaging across all models having $\Delta AIC_c<2$, weighting parameter estimates for each model by the model's Akaike weight and summing the weighted estimates (Burnham & Anderson 2002).

Results

We sampled and AIV-tested a total of 8414 free-living wildfowl of 19 species, of both Eurasian (32%) and Afro-tropical (68%) origin (Table A4.S2). AIV were detected by RRT-PCR in 278 birds (3.3%, 95% confidence interval (CI) 2.9-3.7%). AIV were detected in almost all countries and in all species for which more than 31 birds were sampled. Prevalence was highly variable between species, sites and sampling occasions, reaching up to 14.7% (CI 10.6-19.9%) in Garganey in Mauritania in February 2006. Detailed results of AIV subtypes detected and isolated are presented in Table S3.

The study sites varied greatly in abundance and composition of the local wildfowl community as well as climatic conditions (Table A4.S1). Local wildfowl abundance ranged from a few thousand birds to about a million birds, with densities reaching up to 1500

birds/km². The proportion of Eurasian wildfowl varied between 0-97% according to sites and seasons. The study sites stretched over four aridity classes (from arid to humid), with mean and maximum annual temperatures ranging between 19–28°C and 26–36°C respectively.

We tested the relative influence of six ecological factors on AIV prevalence, including climatic conditions, species traits, host density, influx of Eurasian wildfowl, timing relative to seasonal congregation and to arrival of Eurasian migrants, as well as three potential nuisance parameters (see *Materials and Methods*). A list of variables associated with each ecological factor tested in our study is summarized in Table A4.S4.

The initial selection among alternative variables associated with the same ecological factor, and tested successively by permutation, indicated that the best predictors of AIV prevalence were: annual PET ($\Sigma\omega_i$=0.39) compared to the six other climatic variables, wildfowl community density ($\Sigma\omega_i$=0.92) compared to sampled species density ($\Sigma\omega_i$=0.08), and species taxonomy ($\Sigma\omega_i$ =0.60) compared to geographic origin ($\Sigma\omega_i$=0.36) and foraging behaviour ($\Sigma\omega_i$=0.04) (Table A4.S5).

The AIC-based selection procedure of the complete set of models indicated that four of the six ecological factors tested were important to explain the variation of AIV prevalence in wildfowl (Table A4.S6). The high Akaike importance weights of species taxonomy ($\Sigma\omega_i$ of the models with this factor=0.97), wildfowl community density ($\Sigma\omega_i$=0.98) and the timing relative to arrival of Eurasian migrants ($\Sigma\omega_i$=0.89) indicate that they occurred in a majority of high ranking models. Seasonal congregation of wildfowl estimated from the timing relative to onset of the wet season ($\Sigma\omega_i$=0.60) was also relatively well supported as an important variable across all models. Inclusion of variables associated with the influx of Eurasian wildfowl (% Eurasian wildfowl species, $\Sigma\omega_i$=0.35) or climatic conditions (annual PET, $\Sigma\omega_i$=0.44) received

much less support from the data. Finally, controlling for year ($\Sigma\omega_i$=0.91) and (to a lesser extent) for sample type ($\Sigma\omega_i$=0.54) was important.

Across the full set of models, the best supported overall model (AIC_c=401.9, ΔAIC_c=0) was one that included all four of these factors, as well as the two nuisance parameters year and sample type (Table A4.S7). This model fitted the data adequately (lack-of-fit test P=0.85). Variations in the level of infection (i.e., in observed prevalences) were relatively well predicted by this model, but the absence of infection (i.e. null prevalences) were poorly predicted (Figure A4.S1). This is likely related to detection limit associated with small sample size in some sampling occasions: infected birds may have been present, but at prevalence below the level of detection of the study (Hoye et al. 2010).

All the other five models that received substantial support from the data (ΔAIC_c<2) included the factors of species taxonomy, wildfowl community density and timing relative to arrival of Eurasian migrants, as well as the timing relative to the onset of wet season for a majority of these models (Table A4.S7). Some of these models also contained factors of low relative importance (% Eurasian wildfowl species, annual PET) as seen by their low Akaike weight across all models.

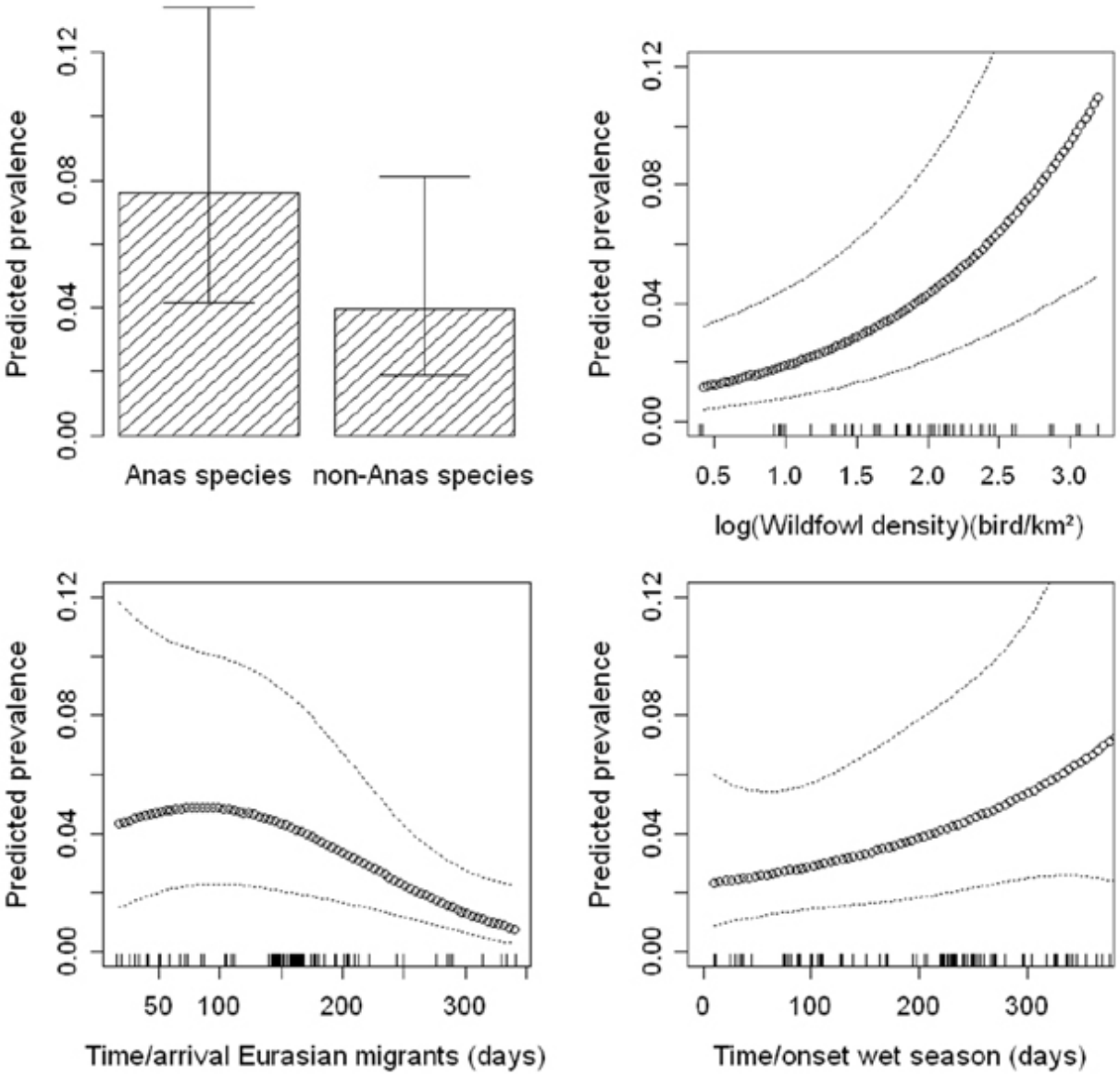
The coefficients of the parameter estimates averaged from the six top models are given in Table A4.S8. AIV prevalence was higher in *Anas* species than in non-*Anas* species and positively associated with the density of the wildfowl community (Figure A4.2). Seasonal variations in AIV prevalence were related to timing relative to both the arrival of Eurasian migrants and the onset of the wet season: prevalence was high and slightly increased during the first half of the wintering period of Eurasian migrants, after which it decreased. Prevalence also progressively increased through the wet season and into the dry season, peaking in the late dry season.

Discussion

Our results show that at a continental scale, the prevalence of AIV infection in wildfowl is strongly influenced by several ecological factors. These factors operate at different levels of ecological organization (host population, community and species) and through environmental drivers. In addition, our results strongly support the hypothesis of an endemic cycle of AIV in Afro-tropical ecosystems rather than a seasonal introduction by Eurasian migratory waterbirds and spillover to African wildfowl. The overall prevalence of AIV found in this study (3.3%) is comparable to AIV detection rates reported in other large-scale studies using similar RRT-PCR screening methods (Munster et al. 2007). AIV were found circulating in all Afro-tropical regions, in all seasons and in all wildfowl species for which a significant number of birds had been tested (Table A4.S2).

In accordance with our initial predictions, our findings suggest that the ecological factors influencing AIV transmission in Afro-tropical regions are derived from specific environmental constraints. The influence of the seasonal congregation of wildfowl on AIV prevalence in the tropics results from the seasonal dynamics of wetland availability instead of the seasonal migration flocking that is typically observed in boreal and temperate regions (Webster et al. 1992, Krauss et al. 2004, Munster et al. 2007). The intensity of such congregation process will vary according to site-specific environments, but could be locally of great importance. For instance, in the Inner Niger Delta in Mali, seasonally flooded wetlands may be up to twenty times as large as the surface area of permanent wetlands (Conway et al. 2009). The increase in local wildfowl density at permanent wetlands in response to the seasonal drying of wetlands likely promotes contact among birds, hence transmission. The positive association that we found between the local wildfowl density and AIV prevalence between sites also supported the hypothesis of a density-dependent transmission process.

Figure A4.2: *Effect plots of the four ecological factors identified as influencing the variation of AIV prevalence in wildfowl in Afro-tropical regions. Plots of predicted prevalence (95% CI) were generated from the highest rank model presented in Table A4.S7. Data points are plotted as rug plots.*



We found no evidence for an influence of climatic constraints on AIV prevalence. Afro-tropical regions are characterised by mean monthly temperatures >20°C in all months (except in African highlands). By contrast, boreal and temperate regions are characterised by mean monthly temperatures <20°C during at least 8 months per year. Temperatures in most Afro-tropical wetlands may be over a threshold where high temperatures throughout the year limit the duration of virus persistence in the environment. In such a context, the environment may have only a minimal role as a source of infection. In African highlands, however, the existence of a cold season may be compatible with the longer-term persistence of viruses in the environment.

Foraging in surface water, typical amongst dabbling wildfowl, or morphological traits associated with filtration of food particles, have been proposed to promote environmental transmission in wildfowl (Olsen et al. 2006, Munster et al. 2009, Hill et al. 2010). In our study, foraging behaviour was a poor predictor of species variation in prevalence. This finding is consistent with a predominant bird to bird transmission of AIV in the tropics rather than a water-borne transmission.

Our findings indicate that the dynamics of AIV infection in wildfowl in Afro-tropical regions are driven by a density-dependent rather than an environmental transmission process. In temperate regions, by contrast, mathematical models suggest a greater role for indirect transmission of AIV from an environmental reservoir than for density-dependent direct transmission (Roche et al. 2009, Rohani et al. 2009). Such differences in the predominant routes of transmission between temperate and tropical zones have also been suggested for human influenza viruses (Lowen and Palese 2009), in response to specific climatic constraints in the tropics. Since the aerosol transmission of human influenza viruses is highly sensitive to humidity and temperature, transmission is considered to occur predominantly by an aerosol

route during the winter in temperate regions, and mainly through direct or indirect year-round contact in the tropics.

Interestingly, most of our initial predictions associated with a seasonal introduction and a spillover of AIV from Eurasian migratory waterbirds were not supported by our data. Species taxonomy (*Anas* versus non-*Anas* species) was a better predictor of species variation in prevalence than geographic origin. Overall, *Anas* species had higher prevalence than non *Anas* species (Figure A4.2), but the prevalence rates of Eurasian *Anas* species (4.9%, CI 4.2-5.8%) and African *Anas* species (5.2%, 4.1-6.5%) were similar (proportion test, $\chi^2_1=0.11$, n=4005, p>0.3). An ancestral co-evolution between AIV and their main host, i.e. *Anas* species, may have resulted in more efficient virus binding and replication in these species (Webster et al. 1992, Munster et al. 2009). In addition, variations in AIV prevalence were poorly related to the percentage of Eurasian wildfowl in the local wildfowl community and AIV was found circulating in seasons and regions when and where Eurasian wildfowl were either abundant or absent.

Several authors have proposed the existence of a north-south decline of AIV prevalence in migratory wildfowl, resulting from a progressive increase in the level of population immunity as birds migrate southward in autumn (Webster et al. 1992, Krauss et al. 2004, Guberti et al. 2007, Munster et al. 2007). A strong seasonal decline in prevalence is commonly observed in wildfowl during autumn in boreal and temperate regions, up to a basal level during northern winter months (generally $\leq$1%; Webster et al. 1992, Munster et al. 2007). By contrast, we found a relatively high prevalence in Eurasian wildfowl from January to March in sub-Saharan Africa (4.9%, CI 4.2-5.8%) with no seasonal decline during their wintering period. This pattern suggests that the level of population immunity in Eurasian wildfowl may remain relatively low throughout the wintering period in sub-Saharan Africa.

The most abundant Eurasian wildfowl wintering in sub-Saharan Africa, Garganey, may have a low exposure to previous AIV infection before arriving in Africa. This species is an earlier and longer-distance migrant than other Eurasian wildfowl (Scott and Rose 1996), with a substantial passage over North Africa in August and September (Zwarts et al. 2009). Moreover, Garganey usually moult in small numbers and do not form large flocks during migration (Zwarts et al. 2009). Garganey may thus escape the major peak of AIV transmission experienced by other wildfowl in autumn in Eurasia. The influence of such migratory behaviour on AIV prevalence has been proposed for another early migrant in North America, the Blue-winged Teal *Anas discors*, for which high prevalence (c. 10-15%) has been consistently reported during winter (Hanson et al. 2005, Ramey et al. 2010).

The prevalence of AIV in wildfowl in our data was higher during the half of the year when Eurasian migrants winter in Afro-tropical regions (c. September-March) than when they were absent from the continent. The presence of Eurasian migrants may influence the local dynamics of AIV transmission in different ways in regions north and south of the equator. In the northern Afro-tropical regions, the arrival of Eurasian wildfowl constitutes a drastic increase in local wildfowl abundance but may also provide a large population of susceptible birds if they have escaped previous AIV infections. Once on their African wintering grounds, Garganeys are highly gregarious. The first months of their wintering period however coincide with the end of the wet season when the area of wetlands is maximal. Eurasian and Afro-tropical wildfowl progressively congregate in shared wetlands as wetlands dry down. Prevalence may thus remain relatively constant throughout the wintering period. Eurasian wildfowl probably leave Africa before an increase in their population immunity reduces transmission rate and prevalence. High AIV prevalence was accordingly found on several occasions in Garganey at the end of their wintering period (February and March) in West

Africa (14.7%, CI 10.6-19.9% in Lake Aleg, Mauritania in 2006; 14.0%, CI 9.0-21.4%, and 15.1%, CI 7.6-27.1%, in the Senegal Delta in 2006 and 2008 respectively).

Alternatively, in African wintering grounds, Eurasian wildfowl may be susceptible to subtypes distinct from those that predominated during their migration across Eurasia (Hanson et al. 2005). Only a few viruses were characterised during our study preventing exploration of this assumption. It is however notable that the AIV subtypes isolated in Afro-tropical regions do not belong to the most common subtypes reported in wildfowl in Europe (Munster et al. 2007).

Eurasian wildfowl do not generally winter in the southern Afro-tropical region, where host species of Eurasian origin are limited to other migratory waterbirds, including shorebirds. A much lower prevalence has been reported globally in non-wildfowl species (≤2%, Olsen et al. 2006, Krauss et al. 2010) suggesting that they play a minor role in the perpetuation of AIV, though locally shorebirds may have a significant role (Krauss et al. 2004, Krauss et al. 2010). Phylogenetic analyses of AIV isolated from wild birds worldwide indicate that inter-continental transfer of AIV genes occurs more frequently in shorebirds than in wildfowl (Dugan et al. 2008). AIV isolated in wildfowl in Southern Africa contained genes whose closest relatives were in viruses found in Europe and Asia, suggesting that migratory shorebirds may constitute a source of AIV introduction in this region (Abolnik et al. 2010).

The predominant role of density-dependent transmission in Afro-tropical regions suggested by our results implies that AIV should be maintained year round through a continuous circulation among wild birds. Maintenance should occur at a relatively low prevalence throughout the year to slow down the controlling effect of herd immunity. In the Afro-tropical context, the ecological factors positively associated with AIV prevalence have a slow seasonal dynamic. The seasonal congregation of wildfowl results from a progressive

gathering of birds throughout the dry season, and the extended breeding seasons result in a low turnover of young susceptible birds in host populations. In accordance, the seasonality of influenza infection we measured in our study was less pronounced (seasonal peak in prevalence ≤15%) than in Europe (25%, Wallensten et al. 2007) or North America (40%, Krauss et al. 2004). Analogous differences in human influenza seasonal patterns between temperate and tropical regions have also been reported, with a low background influenza activity throughout the year in the tropics compared to high seasonal epidemics in temperate countries (Lowen and Palese 2009).

Regional differences in the composition of the wildfowl community may also determine the background level at which AIV are perpetuated in tropical Africa. *Anas* species likely play a major role among wildfowl in the perpetuation of AIV, as their consistently higher prevalence suggests (Olsen et al. 2006, Munster et al. 2007). These species are, however, not homogenously distributed among Afro-tropical regions. In West Africa, *Anas* species are represented almost only by Eurasian migratory ducks (Scott and Rose 1996), and therefore there are almost no *Anas* ducks during c. 6 months of each year. African *Anas* ducks are abundant year round in both Eastern and Southern Africa, but Eurasian *Anas* ducks are largely absent in the regions south of the equator. As a consequence, AIV may be perpetuated annually at a background level that decreases sequentially between Eastern, Southern and West Africa.

AIV may be perpetuated at a continental scale through a meta-population process between Afro-tropical regions. There is an asynchrony in the timing of rainfall and associated seasonal ecological drivers influencing AIV transmission between regions north and south of the equator. This may create a network of complementary areas across the Afro-tropical regions with temporarily suitable conditions, in terms of host density and production of susceptible juvenile birds, for AIV maintenance. Local declines or extinction in AIV

circulation may be balanced by a seasonal re-introduction through exchanges of host populations and AIV dispersal within regions (Chen and Holmes 2009). Waterbirds in Africa make a wide range of movements, largely based on rainfall patterns and including some nomadic movements. However, the extent and frequency of intra-African waterbird migrations remain poorly understood, limiting our abilities to predict the level of interaction between regional AIV cycles.

Finally, our results support the hypothesis that Afro-tropical regions may contribute to the global year round perpetuation of AIV by providing a seasonal shelter for the maintenance of AIV in wildfowl (Webster et al. 1992). With prevalence remaining relatively high in sub-Saharan Africa throughout the northern winter period, Eurasian migratory waterbirds may re-introduce some AIV into temperate and boreal regions during their spring migration. In our study, some Garganey were found infected at high prevalence at the time they leave their Sahelian wintering areas (February-March), but also during spring migration in North Africa as they return to their Eurasian breeding grounds (5.7%, CI 1.2-18.6%, in Egypt in April, Table A4.S9). Up to now, however, no African lineage of AIV has been reported in birds in Europe, though few African viruses have been characterised.

This study demonstrates the value of integrating ecology and epidemiology for understanding complex multi-host epidemiological systems (Stallknecht 2007). Our results also demonstrate the importance of obtaining detailed data across a wide range of environmental conditions and host communities (Tompkins et al. 2010). In the development of general models of avian influenza dynamics, it is clear that some mechanisms (e.g. environmental transmission) may have been over-emphasized in the peer-reviewed literature, while others (e.g. responses to seasonal fluctuations in habitat) have been largely ignored because their influence is less obvious in temperate regions. As our analysis shows, research

at the interface between ecology and epidemiology could benefit hugely from inter-group data sharing and detailed empirical analyses of geographically diverse data sets.

Supporting Information (not displayed in the thesis)

Additional Supporting Information may be found in the online version of this article:

Table A4.S1: List of sites ranked by latitude where wildfowl were sampled during this study. Estimates of abundance and composition of the local wildfowl community at the time of sampling are presented for each site, as well as the mean annual climatic conditions. Range values (min-max) are presented for sites sampled on several occasions. These data represent only values estimated for the periods when sampling was conducted (2006-2009), and more extreme values occur.

Table A4.S2: Prevalence of avian influenza viruses in Eurasian (a) and Afro-tropical (b) wildfowl species sampled in this study and tested by real-time RT-PCR.

Table A4.S3: Table S3. List of AIV subtypes a) detected by conventional RT-PCR specific for H5 or H7, or b) isolated in embryonated SPF chicken eggs.

Table A4.S4: Definition of the explanatory variables associated with the ecological factors examined in our analyses.

Table A4.S5: Selection of alternative explanatory variables associated with the same ecological factor, tested successively by permutation in a set of global models. Bold text depicts the selected explanatory variables based on their relative importance, estimated by summing the normalized Akaike weights ($\Sigma\omega_i$) over the subset of model which contained that variable.

Table A4.S6: Relative importance of selected explanatory variables explaining variations in AIV prevalence in wildfowl in Afro-tropical regions estimated by summing their Akaike weights ($\Sigma\omega_i$) over the subset of models that contain that variable.

Table A4.S7: Selection statistics of the top ten candidate beta-binomial models describing the variations in AIV prevalence in wildfowl in Afro-tropical regions. Models are ordered by AIC_c rank and the six best-supported models ($\Delta AICc<2$) are highlighted in bold.

Table A4.S8: Model-averaged parameter estimates of the relationships between AIV prevalence (logit) in wildfowl and four ecological factors and two nuisance parameters identified as important predictors.

Table A4.S9: Prevalence of avian influenza viruses in Eurasian wildfowl sampled in one additional sampling site in North Africa (Nil delta, Egypt) in April 2008.

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