Aus der Abteilung für Wildtierkrankheiten
im Leibniz Institut für Zoo und Wildtierforschung
im Forschungsverbund Berlin e.V.
eingereicht beim Fachbereich Veterinärmedizin
der Freien Universität Berlin

Seroprevalence of Influenza A viruses in wild Mammals

Inaugural-Dissertation
zur Erlangung des Grades einer
Doctorin der Veterinärmedizin (Doctor
medicinae veterinariae)
an der Freien Universität Berlin
vorgelegt von
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Berlin 2019

Journal-Nr.: 4135

Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan	Univ.-Prof. Dr. Jürgen Zentek
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Deskriptoren (nach CAB-Thesaurus):

- Equidae
- Donkeys
- Influenza a virus
- Epidemiology
- Disease vectors
- Public health
- Transmission
- Diet
- Mammals
- Africa
- Serology
- Exposure
- Antibodies
- ELISA

Tag der Promotion: 09.12.2019

In memory of my father

Dedicated to my family and friends who supported me all the way through! With you at my side, I can achieve anything!

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Mongolian Khulan (*Equus hemionus hemionus*)
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This thesis is based on following manuscripts:

Soilemetzidou, Eirini-Sanatana, Erwin De Bruin, Mathias Franz, Ortwin H K Aschenborn, Guus F Rimmelzwaan, Ruud Van Beek, Marion Koopmans, Alex D Greenwood, and Gábor Á Czirják. 'Diet May Drive Influenza A Virus Exposure in African Mammals'. The Journal of Infectious Diseases, 6 March 2019. https://doi.org/10.1093/infdis/jiz032.

Soilemetzidou ES, Bruin E de, Eschke K, et al. Bearing the brunt: Mongolian khulan (Equus hemionus hemionus) are exposed to multiple influenza A strains. Journal of General Virology 2019; (submitted)

Soilemetzidou S-E, Greenwood AD, Czirják GÁ (2018) VERY LOW INFLUENZA A VIRUS PREVALENCE IN CERVIDS IN GERMAN NATIONAL PARKS. *Journal of Zoo and Wildlife Medicine* 49(1): 252–254.

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Abbreviations

IV	Influenza viruses
IAV	Influenza A viruses
AIV	Avian influenza viruses
HA	Hemagglutinin
NA	Neuraminidase
EIV	Equine influenza viruses
AI	Avian influenza
HPAIV	Highly pathogenic avian influenza virus
LPAIV	Low pathogenic avian influenza virus
HP	Highly pathogenic
LP	Low pathogenic
RT-PCR	Reverse transcription polymerase chain reaction
HI	Hemagglutination inhibition assay
PM	Protein microarray
VNT	Virus neutralization test
SRH	Single radial hemolysis
OIE	World Organization for Animal Health
WHO	World Health Organization

Chapter 1
General Introduction

1.1 Classification

Influenza viruses (IV) are negative-stranded RNA viruses with segmented genomes which replicate in the nucleus of infected host cells. They belong to the family Orthomyxoviridae and are classified in seven genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Influenzavirus D, Thogotovirus, Isavirus, and Quaranjavirus. They are important viruses in veterinary medical and human health, with a diverse host range and worldwide distribution. Influenzavirus A has a broad host spectrum, with wild waterfowl being known as the natural hosts. They infect many species, such as humans, swine, equids, and poultry among the most important (Webster et al., 1992). Influenzavirus B commonly infects humans and seals (Matsuzaki et al. 2004; Osterhaus et al. 2000), while Influenzavirus C infects humans and pigs (Matsuzaki et al. 2005; Kimura et al. 1996). Cattle have been proposed to be the natural host of Influenza virus D, while it has been found to infect swine, sheep, goats and camels (Ferguson et al. 2015; Salem et al. 2017). Thogoviruses, in contrast to most of the Orthomyxoviridae, are arboviruses, and Rhipicephalus ticks usually transmit them. They infect a wide variety of animals, and some of the subtypes are infectious to humans as well. Isavirus is a fish pathogen and causes anemia in salmons (Kibenge et al. 2006). Quaranjavirus has been isolated from ticks and birds, although it may infect humans as well (Presti et al. 2009). Influenza A viruses are the focus of this thesis.

1.2 Viral structure and transmission

The natural hosts of influenza A viruses (IAV) are presumed to be wild birds of the orders Anseriformes (ducks, geese, and swans) and Charadriformes (gulls, terns, and shorebirds). However, they have the ability to infect other avian groups including poultry and non-avian species such as humans, horses, swine and marine mammals establishing endemic infections. IAV subtype is determined by characterizing the hemagglutinin (HA) and neuraminidase (NA) viral surface proteins subtypes. There are currently 18 different HA, and 9 NA described, with HA 17 and 18 being found only in bats (Tong et al. 2013). All other subtypes are found in bird species.

Influenza viruses are enveloped viruses, and the virion is roughly spherical. The outer layer is a lipid membrane, in which three external proteins can be found —HA and NA, and also the M2 protein. Envelopes lipid membranes derived from the host cell membrane during viral budding which helps the viruses to avoid the host immune system (Schaap et al. 2012). Furthermore, the glycoproteins on the surface of the envelope play an important role in viral entry and membrane fusion. Between the envelope and the viral capsid is the Matrix protein (M1). The internal proteins include the nucleoprotein (NP), and the polymerase complex. This complex contains the polymerase basic

protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). An additional protein, nuclear export protein (NEP), which is composed of the non-structural protein 1 and 2 (NS1 and NS2 respectively), is also part of the virion (Figure 1) (Bouvier and Palese 2008). Influenza viruses are classified as low- and highly pathogenic (LPAIV and HPAIV respectively), depending on their ability to cause disease and mortality in chickens. This ability does not refer to human disease, although both LPAIV and HPAIV can cause severe disease in humans.

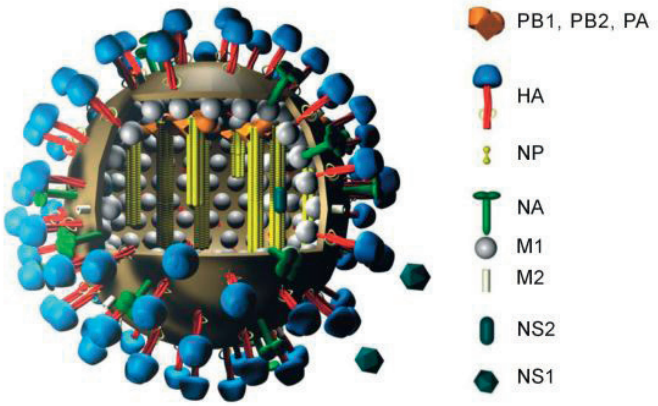

Figure 1: 3D modell of Influenza virus with all of it's proteins. M.Eickmann

The transmission route of Avian influenza viruses (AIV) occurs mainly via a fecal-oral route. The virus replicates in the epithelial cells of the intestinal tract, and is shed via the feces, often contaminating water, which then acts as a viral vector (Hinshaw et al. 1979). Water transmission is supported by numerous studies, which have shown the persistence of influenza viruses on water surfaces (Dalziel et al. 2016, Keeler et al. 2014). Generally, the shedding estimation time in birds is between 2-6 days (Henaux and Samuel, 2011). Another critical factor for influenza transmission is the ecology of their natural host. Migration is considered as one of the main factors influencing the transmission of the virus. Migratory waterfowl are known to carry pathogens, which during their migration, in stop-over sites, in breeding and overwintering sites, will be transmitted to other populations (Olsen et al. 2006). Aggregation of different species over time and space may also influence the prevalence, transmission, and evolution of AIV (Garamszegi and Möller 2007). Variation in the prevalence and subtypes of influenza in different species may also be an additional factor of influenza ecology and evolution(Munster et al. 2007).

An additional factor that impacts influenza ecology and epidemiology is the potential for genetic reassortment and mutation, so called antigenic shift and drift. IAVs have segmented genomes and a polymerase, which lacks proofreading ability, leading to high rates of mutation. Genes from different strains can reassort when one cell is infected with two or more different strains

(Clancy, 2008). Pigs are considered mixing vessels, where human and AIV reassortment occurs, because of their susceptibility to both viruses. In addition to pigs natural susceptibility, intensive and backyard farming, especially in Southeast Asia and China bring waterfowl and domestic pigs in close contact, providing fruitful ground for viral exchange. Although there is evidence for antigenic shift in pigs, it is unclear if swine have contributed to any pandemic (Ito et al. 1998). Nevertheless, mutations and reassortment, could possibly help influenza acquire new hosts, crossing species barriers and enhancing pathogenicity (Mehle et al. 2012). Furthermore, although all genes of IAV contribute to pathogenicity and host range, the HA and polymerase seem to play the most important role (Ping et al. 2010).

1.3 Influenza A virus through the history

The history of influenza virus epidemics extends back to the time of Hippocrates in 412 BC in ancient Greece, when he described a disease with symptoms consistent with influenza infection in his texts Corpus Hippocraticum. Although there is no certainty as to the actual disease, it is often considered the first influenza epidemic description (Pappas et al. 2008). Considering that the diagnosis came from reported personal observations without any virological evidence, the causative agents in past disease pandemics in general have not been confirmed with few exceptions where ancient DNA could be obtained (Guellil et al 2018). Despite these difficulties epidemiologists generally agree that the 1580 outbreak, which started in Asia and spread to Africa and Europe, is the earliest historically recorded epidemic (Pyle et al. 1986). There were probably at least six subsequent pandemics in the 18th century, followed by possibly four more during the 19th century (Potter 2001). The most deadly one known was the 1918 influenza pandemic, colloquially know as Spanish flu (H1N1), which killed almost 50 million people (Johnson and Mueller 2002). In 1957 a new influenza virus emerged, H2N2, triggering the first modern Asian flu pandemic, followed by the pandemic of 1968, where a new subtype, H3N2 appeared in Southeast Asia. The H3N2 virus was a reassortment of the 1957 neuraminidase and a new hemagglutinin. The last pandemic of the 20th century circulated in the Union of Soviet Socialist Republics between 1977-78, followed by the first pandemic of the 21st century, H1N1, which started in the US (Kilbourne 2006). The success of the virus is primarily due to antigenic drift and shift, which makes emerging strain prediction nearly impossible and makes influenza one of the most significant concerns for human and animal health.

1.4 Influenza A virus hosts

1.4.1 Birds

Generally, the birds shed virus for 2-6 days (Henaux and Samuel, 2011). Wild aquatic birds play a significant role in influenza transmission and ecology, not only because they are the natural hosts, but also due to their annual migration patterns. There are currently three major migratory flyways that have been identified; the American flyway, the African-Eurasian flyway and the East Asian-Australasian flyway. Mixed infections and reassortment are common in aquatic wild birds, due to the overlapping of breeding and wintering grounds (Wang et al. 2008). The extent to which wild resident birds play a role in influenza ecology is unclear. Evidence suggests that wild birds that do not belong to the family of Anseriformes or Charadriformes could also play a maintenance and transmission role on the influenza ecology (Caron et al. 2014; Caron et al. 2017; Hill and Runstadler 2016).

1.4.2 Mammals

1.4.3.Horses

Equine influenza virus (EIV) is one of the most common respiratory diseases in equids. It infects different equids (horses, mules, donkeys) and shows high morbidity and low mortality. EIVs have been observed simultaneously with human influenza for hundreds of years (Morens and Taubenberger 2010). Domestication of horses and other species, and the emergence of pandemic influenza viruses have been correlated (Shoham 2011). The first EIV isolated was A/H7N7 (or Equine-1 subtype) in 1956 (Tumova 1980). The second subtype of EIV appeared in 1963, the H3N8 (or equine-2 subtype) (Waddell et al. 1963), which remains the most critical pathogen in equids, circulating worldwide. Although H7N7 was isolated for the last time in 1979 (Webster 1993), there are still sporadic reports of serological evidence in Central Asia and Africa (Singh 1995; Olusa et al. 2010). EIV was always viewed as strictly species-specific. A big game changer, was the jump of H3N8 equine influenza to dogs, around 2004 in the USA (Crawford et al. 2005). This case was the first demonstration of the zoonotic potential of equine influenza H3N8. Furthermore, EIV was isolated from a Bactrian camel in Mongolia (Yondon et al. 2014), and there is some serological evidence for potential infection of humans (Xie et al. 2016). H3N8 has an avian origin (Joseph et al. 2017) and preferably binds to avian-like α-2,3-sialic receptors, which are abundantly expressed in the upper respiratory tract of horses. The expression of these receptors may make equids more susceptible to avian-like influenza viruses, such as HP H5N1 (Abdel-Moneim et al. 2010).

1.4.4 Other mammals

AIV is known to have zoonotic potential, which is of great concern for human and animal public health. Some subtypes have crossed the species barriers and have managed to establish endemic infections, like equine and canine influenza viruses. A fundamental aspect of the establishment of an infection is the presence of the virus long enough in a population so that the virus can adapt to the new host. In other cases, the spill-over effect is sporadic, like H5N1 infection in captive tigers and leopards (Keawcharoen et al. 2004). In such cases, transmission is possible, but the virus is not present long enough so that adaptation and establishment can take place. However with the current rapid anthropogenically driven change of ecosystems, animals are being forced to live in more confined spaces, increasing the contact between natural and intermediate hosts, which might increase the chance for viral adaptation and species cross-transmission. Recent influenza infection events display a lack of influenza host specificity. Various wild and domestic mammalian species have been infected with different strains of AIV. In addition to H5N1 which has been involved in infection of various mammalian species (Swayne et al. 2017), H1N1 was detected in captive cheetahs (Crossley et al. 2012), Giant pandas (Li et al. 2014) and domestic cats and dogs (Au et al. 2013; Jang et al. 2017). Although the establishment and mammal to mammal transmission is still unknown, the evolution and establishment of the virus in new hosts, poses a considerable risk.

1.5 Influenza surveillance

Influenza A virus surveillance is of fundamental importance for both animal and human health. The surveillance activities can be passive or active, based on data collection method, and can be managed by national or the private sector. Active surveillance is based on data collection from targeted sampling, including sentinel surveillance. Passive surveillance gathers data from health authorities reporting disease cases. Data collected are used to inform risk assessment and management of human and animal health in the respective country. Although most surveillances are comprehensive, they are often geographically biased and focused on HPAIV, which generate the greatest economic loses (von Dobschuetz et al. 2014). Surveillance and diagnosis of influenza viruses focus on the detection of the virus or viral-specific antibodies. The most sensitive detection and characterization of the strain can be accomplished by oral or rectal (cloaca) swabs, using reverse transcription polymerase chain reaction (RT-PCR) or viral isolation, depending on the species and sample availability. While viral characterization has a high positive monitoring value, these techniques share some disadvantages. Viral shedding typically lasts only for a couple of days. Therefore, serological evidence of exposure gives a broader window for detection of infection. Furthermore, serologi-

cal methods are relatively inexpensive, and some of them can be used in the field, without the need for sample transportation and processing prior characterization (Swayne, 2017). Serological methods used for wildlife species may have some disadvantages as well. Most of the tests were developed and validated on domestic species. The same tests might not work with specificity or sensitivity on wildlife. Differences in the exposure and immune response of the animal, as well as differences in pathogen strains and serovars might alter the outcome of the assays. Furthermore, the absence of antisera to IgG or IgM for wild- or exotic species has to be overcome (Gardner et al 1996; Gilbert et al 2013). Cut-off values are normally set under laboratory conditions and for vaccination. Therefore, interpretation of the results and discrimination of positive or negative animals might not be conclusive e (Gilbert et al 2013).

A further disadvantage of the current influenza surveillance is the prioritizing of species. World Organization for Animal Health (OIE) has emphasized to the surveillance of HPAIV in wild birds. The adaptation of the IV in novel host species suggests that ecological, behavioral, and genetic barriers have previously been crossed several times. Therefore, a more comprehensive and extensive surveillance of mammalian species may provide added information regarding influenza epidemiology and ecology. There is a need for greater understanding of influenza host physiology, transmission, ecology and the role that wildlife plays in these cycles. Wildlife surveillance is a challenging field, often associated with a limited number of samples, obtained mostly opportunistically. Therefore, interdisciplinary work, bringing together researchers and wildlife professionals will be needed to unravel the role of wildlife in AIV ecology. Targeting more influenza viruses and not only HPAIV, which are of a greater interest to human and public health, but also screening for other subtypes, will strengthen our knowledge and understanding of the epidemiology and ecology of AIV.

1.6 Aim of the study

The aim of this study was to determine IAV exposure and diversity in wild mammals. Although IAV in wild mammals may currently not necessarily pose a direct threat to human and public health, it is of importance to understand the role that those animal play in the context of influenza ecology and epidemiology. Non-avian wildlife has been previously identified to be infected with different IAV subtypes, such as wolves and gazelles in China (Wei et al. 2016), raccoons in Japan (Horimoto et al. 2011), or crocodiles in Florida (Davis and Spackman 2008). Consequently, a prediction of if and when AIV may change pathogenicity, host range, and adaptation is currently impossible. A better understanding of the prevalence and diversity of influenza subtypes in different species is needed for future risk assessment. To achieve

that, factors such as diversity of species in a defined place, interactions between avian and non-avian wildlife as well as the ecological changes that occur in their natural environments need to be understood. We hypothesized, that wild mammalian species with close contacts to IAV reservoirs or domestic animal species, could represent an unexplored influenza reservoir. To address this hypothesis, 16 mammalian species in 3 different continents where serologically analyzed for presence and diversity of influenza viruses.

1.6.1 Chapter 2: Namibia, Africa

I conducted an epidemiological survey among different wild mammalian species from Namibia. The goal was to screen for the presence of AIV antibodies in species with an unknown role in influenza ecology and transmission. For that purpose, one hundred and eleven sera samples from 14 different wild mammalian species from Etosha National Park in north-central Namibia and the Caprivi region in northeastern Namibia were analyzed (Figure 2). Samples were screened for the presence of IAV antibodies, using a protein microarray and hemagglutination inhibition assays. Furthermore, species where divided into categories and the effect of sociality, diet and phylogeny was investigated. This study is described in detail in Chapter 1.

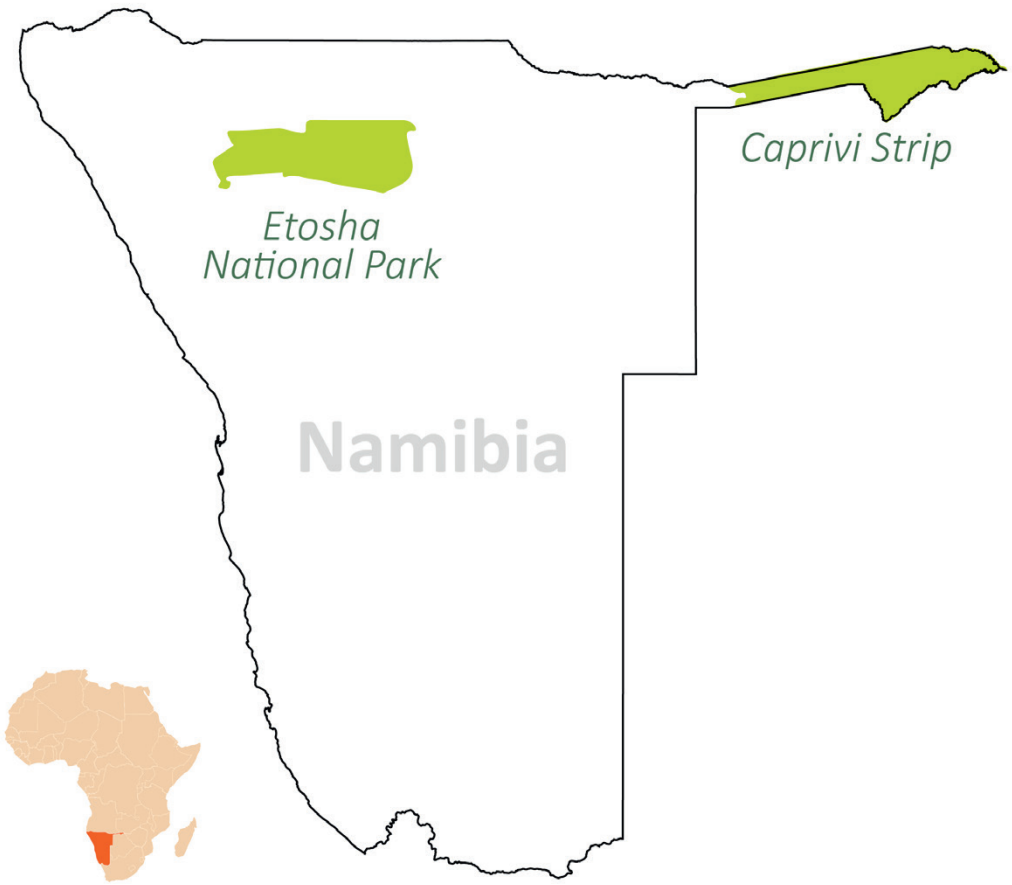

Figure 2: The green areas represent the two National Parks were the animals were sampled.

1.6.2 Chapter3: Mongolia, Asia

Twenty-one serum samples from Asiatic wild Asses were analyzed by using a protein microarray technique, HI, Virus Neutralization Test (VNT) and Single Radial Hemolysis Assay (SRH) for the presence of EIV and other IAV antibodies. The free ranging animals were from the Southern Gobi Desert in Mongolia (Figure 3), sharing the same pastures with a large number of domestic horses. The aim of the study was to investigate the exposure of wild equids to equine and other avian influenza viruses. This study is described in Chapter 2.

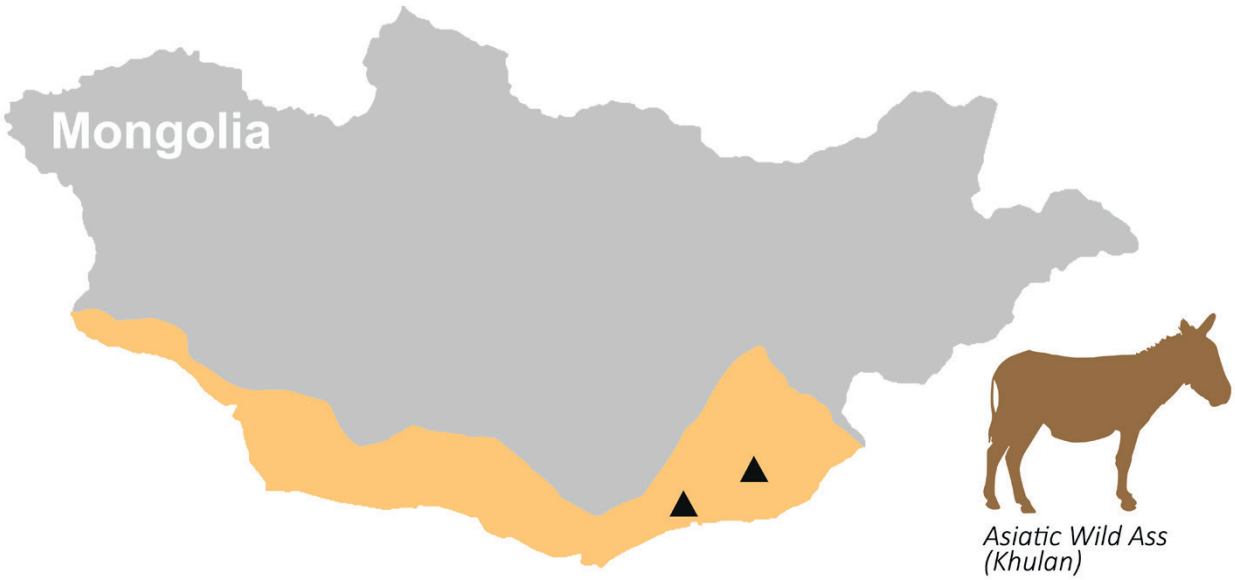

Figure 3: The orange color represents the Gobi Desert and distribution of khulans. The triangles represent the sampling sites of the animals.

1.6.3 Chapter 4: Germany, Europe

Serum samples from 3 different Cervidae species (Roe-, Red- and Fallow deer) were collected from 6 National Parks of Germany (Figure 4), to investigate their exposure to different IAVs which may pose a risk to human and domestic animal health. One hundred and thirty seven serum samples were screened for antibodies using the ID Screen® Influenza A Antibody Competition Multi-Species Enzyme-Linked Immunosorbent Assay (ELISA) kit, as described in chapter 3.

Figure 4: The map shows with green the 6 National Parks were the animals were sampled.

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Chapter 2

Diet May Drive Influenza A Virus Exposure in African Mammals

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Received 19 September 2018; editorial decision 3 December 2018; accepted 24 January 2019; published online March 6, 2019.
Presented in part: 67th Annual International Wildlife Disease Association Conference, St.Augustine, FL, 5–10 August 2018. [a]A. D. G. and G. A. C. contributed equally to this work.
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The Journal of Infectious Diseases® 2019;XX(XX):0–0

DOI: 10.1093/infdis/jiz032

Background. Influenza A viruses (IAVs) represent repeatedly emerging pathogens with near worldwide distribution and an unclear nonavian-host spectrum. While the natural hosts for IAV are among waterfowl species, certain mammals can be productively infected. Southern Africa is home to diverse avian and mammalian fauna for which almost no information exists on IAV dynamics

Methods. We evaluated 111 serum samples from 14 mammalian species from Namibia for the presence of IAV-specific antibodies and tested whether host phylogeny, sociality, or diet influence viral prevalence and diversity.

Results. Free-ranging African mammals are exposed to diverse IAV subtypes. Herbivores developed antibodies against 3 different hemagglutinin (HA) subtypes, at low prevalence, while carnivores showed a higher prevalence and diversity of HA-specific antibody responses against 11 different subtypes. Host phylogeny and sociality were not significantly associated with HA antibody prevalence or subtype diversity. Both seroprevalence and HA diversity were significantly increased in carnivores regularly feeding on birds.

Conclusions. The risk of infection and transmission may be driven by diet and ecological factors that increase contact with migratory and resident waterfowl. Consequently, wild mammals, particularly those that specialize on hunting and scavenging birds, could play an important but overlooked role in influenza epizootics.

Keywords. Influenza A virus; transmission; disease; diet; mammals; Africa; serology; protein microarray; exposure.

Southern Africa has a large diversity of mammals, with many species closely related to domestic and captive animals known to be susceptible to influenza A virus (IAV) infection. At least 3 different flyways (East Atlantic, Black Sea–Mediterranean, and West Asian–East African), cross the region and are seasonally used by large flocks of migratory birds [1]. Avian influenza viruses are known to circulate among African waterfowl, with reports from Egypt, Southern African countries, and Kenya [2–4].

Water birds among the orders Anseriformes and Charadriformes [5] are considered to be the natural reservoirs for IAVs. Avian influenza viruses have crossed species barriers, establishing endemic infections among a taxonomically limited number of domestic mammals, such as horses and swine [6]. In some cases, this has resulted in subsequent transmission to other mammals, such as from horses to dogs [7]. Among wild animal species, reported influenza virus infections are sporadic and mainly limited to captive animals, such as IAV H5N1 infections in leopards and tigers [8], or IAV H1N1 infection in cheetahs [9]. Such opportunistic infections do not necessarily result in mammal-to-mammal transmission, but there are some exceptions, such as highly pathogenic IAV H5N1 transmission in captive tigers [10]. In contrast to studies in captive settings [11], reports on natural infections of free-living mammalian species are relatively scarce, with some reported infections in wild raccoons and stone martens [12–14]. Serological evidence for natural infections has also been described for sea otters and nonhuman primates, such

Table 1. Species and Number of Individuals Evaluated With the Protein Microarray

Species	Individuals Tested, No.
Black rhino (*Diceros bicornis*)	10
African elephant (*Loxodonta africana*)	9
Springbok (*Antidorcas marsupialis*)	10
Wildebeest (*Connochaetes taurinus*)	10
Brown hyena (*Hyaena brunnea*)	4
Spotted hyena (*Crocuta crocuta*)	9
Honey badger (*Mellivora capensis*)	7
Black-backed Jackal (*Canis mesomelas*)	10
Bat-eared fox (*Otocyon megalotis*)	4
African wild dog (*Lycaon pictus*)	7
Lion (*Panthera leo*)	10
Leopard (*Panthera pardus*)	9
Cheetah (*Acinonyx jubatus*)	4
Caracal (*Caracal caracal*)	8

as gibbons and macaques [15], with most representing direct infections from avian hosts or infection with human influenza virus [15].
Among mammals, transmission routes for pathogens spread via aerosol or an oral-fecal route would likely be confronted with several potential, non–mutually exclusive benefits and challenges to dissemination. The close relationships among the species would likely be reflected in closer relatedness of viral receptors and antiviral defenses, such that a virus able to infect one species may have a higher likelihood of infecting another closely related species, as has been observed with the spread of feline immunodeficiency virus among felids [16] or the transmission of foot-and-mouth disease between African buffalo and impala [17]. Variation in viral receptor distributions among species can alter IAV transmission from birds to mammals or from mammals to birds [18–20]. Transmission subsequently allows for potential reassortment and viral adaptation.

Many animal species form complex social groups, which are known in some cases to promote pathogen transmission [21, 22], sometimes with a devastating outcomes [23]. Many African herbivores form large migratory herds, which can range from a few to several hundred individuals, depending on the season, and can promote both intraspecies and interspecies transmission of pathogens [24, 25]. Several African carnivore species are social (eg, lions, hyenas, and wild dogs), which facilitates pathogen transmission not only within but also among species.

Diet is another source of infection by viruses transmitted by aerosol or the oral-fecal route. Either direct infection by consuming a reservoir or contact with excrement from the reservoir during hunting could promote pathogen transmission [26, 27]. For example, *Streptococcus equi* infection of spotted hyenas due to intake of infected zebra meat has been observed [28]. Although herbivores might be exempt from diet-driven pathogen transmission, sharing common feeding grounds and water sources with the reservoir host could also lead to potential transmission. Pikas, for example, are thought to have been infected with IAV H5N1 circulating in wild birds at common weed-foraging sites [29].

In the current study, we addressed whether phylogenetic relatedness, sociality, or diet could account for IAV infection patterns among free-living African mammals, focusing on Namibia. Specifically we tested the following predictions: (1) closely related species have more similar levels of IAV prevalence and hemagglutination diversity than more-distantly related species, (2) social species are associated with increased IAV infection rates and strain exposure diversity, and (3) consumption of birds is associated with increased IAV infection rates and strain exposure diversity. Using an

IAV antigen array and hemagglutination inhibition assays and 111 serum samples obtained from 14 mammalian species, we determined exposure of these African animals to IAVs of various subtypes.

METHODS

Study Samples

Blood samples were collected between 2009 and 2013 from different mammal species in the context of relocation programs and different research projects running in Etosha National Park in north-central Namibia and in Caprivi Region in northeastern Namibia. All handling of animals was performed by or under direct supervision of the wildlife veterinarian responsible for these areas, ensuring compliance with animal welfare regulations. After blood sampling, serum samples were obtained and stored in liquid nitrogen until they were transported to the Leibniz Institute for Zoo and Wildlife Research in Germany, in full compliance with the Convention on International Trade and in Endangered Species, where they were stored at −80°C.

Serological Exposure and Diversity of IAVs

A modified protein microarray technique was used to test the serum samples for the presence of antibodies against the complete panel of IAV hemagglutinin (HA) subtypes, ranging from H1 to H16 (Supplementary Table 1). Samples were inactivated in a water bath at 56°C for 4 hours, owing to regulations for the testing of animal samples from areas of foot and mouth disease endemicity. Serum samples from 111 animals from 14 species (Table 1) were tested against 2 different secondary antibodies to determine the highest sensitivity per species: protein A conjugated to Alexa Fluor 647 (Molecular Probes, Waltham, MA) and biotinylated protein G (Thermo Scientific, Waltham, MA) in combination with a mouse anti-biotin immunoglobulin G conjugated to Alexa Fluor 647 (Jackson Immunoresearch, Ely, England). The highest sensitivity was detected against protein A, except for springbok and wildebeest samples, which were therefore tested using protein G.

Briefly, 32 recombinant proteins of different IAV antigens were printed onto 16-pad nitrocellulose film slides (Oncyte Avid; Grace Bio-Labs, Bend, OR). All presently known IAV HA subtypes are represented on the array, except for HA subtypes 17 and 18, as they have only been detected in bats. Slides were treated with Blotto Blocking Buffer to avoid nonspecific binding (Thermo Fischer Scientific, Rockford, MA) for 1 hour at 37°C in a moist chamber. After the slides were washed, they were incubated with a 4-fold dilution series, from 1:20 to 1:1280, of the serum samples. After incubation for 1 hour at 37°C, slides were washed and incubated with a 1:500 dilution of the secondary antibody (ie, protein A or G) as described before. A final washing step was done to remove unbound conjugate, after which the slides were dried and scanned using a Powerscanner (Tecan). Spot intensities were

determined, and titer heights were calculated by curve fitting, using R (R Statistical Computing, version 3.1.0; Vienna, Austria). Titers <20 were set to 20.

Hemagglutination Inhibition (HI) Assays

To confirm the protein microarray results, selections were made on the basis of the availability of the probable infecting influenza virus strain to be used in HI assays and the amount of serum available of the animals to be tested. Four honey badger serum samples were tested against influenza virus strains A/ Anhui/001/2013(H7N9) and A/ Mallard/the Netherlands/12/00(H7N3). Four lion sera were tested using 2009 pandemic influenza virus strain A/ California/007/09(H1N1), and 5 jackal sera were tested against strains A/Vietnam /1194/2005(H5N1, clade 1) and A/Turkey/Turkey/001/2005(H5N1, clade 2.2). Serum samples were pretreated with receptor-destroying enzyme during incubation for 16 hours at 37°C, followed by 1 hour at 56°C. Two-fold dilution series starting from 1:20 were made of the pretreated sera, and 4 HA units of virus was added to a final volume of 75 µL. After incubation for 30 minutes at 37°C, 25 µL of 1% turkey erythrocytes was added, and the sample was incubated for 1 hour at 4°C. After 1 hour, hemagglutination patterns were read, and the HI titer was expressed as the highest serum dilution that still completely inhibited hemagglutination. Titers for serum samples testing negative for HI in the first dilution were expressed as <10.

Statistical Analysis

Whereas seroprevalence could be readily calculated for each species, the observed number of HA subtypes per species might be strongly influenced by the variation in sample sizes across species. To control for this variation, we calculated a diversity index for each species that represents the number of HA subtypes that is expected for a sample of 4 individuals (which was the smallest sample size in the data set). For this purpose, we generated for each species all possible subsamples of 4 individuals. The number of HA subtypes for each subsample was calculated averaged across all subsamples to obtain a diversity index. Pagel's lambda [30] was used to investigate the influence of phylogenetic relatedness on influenza virus prevalence and diversity. Pagel's lambda is a tree transformation metric that varies between 0, which indicates the absence of any phylogenetic signal, and 1, which indicates that the distribution of the variable matches a Brownian model of evolution.

A phylogenetic tree was generated based on an alignment of the mitochondrial genome of each species from the National Center for Biotechnology Information database and aligned using only the coding regions in Geneious v9.1.8 software (Supplementary Figure 1). A maximum likelihood tree was calculated using PHYML. Because of missing sequences of honey badger (Mellivora capensis), black-backed jackal (Canis mesomelas), and

brown hyena (Hyaena brunnea), the closely related species European badger (Meles meles), steppe wolf (Canis lupus campestris), and striped hyena (Hyaena hyaena), respectively, were substituted. To estimate Pagel's lambda [30] for each variable we used the packages "phytools" [31] and "geiger" [32]

Table 2. Diet and Social Organization of the Carnivorous Species Tested

Species	Diet	Sociality
Lion	Rarely or never eat birds	Social
Leopard	Rarely or never eat birds	Solitary
Cheetah	Rarely or never eat birds	Opportunistically social
Brown hyena	Rarely or never eat birds	Social
Spotted hyena	Rarely or never eat birds	Social
African wild dog	Rarely or never eat birds	Social
Bat-eared fox	Rarely or never eat birds	Social
Black-backed jackal	Commonly eat birds	Opportunistically social
Caracal	Commonly eat birds	Solitary
Honey badger	Commonly eat birds	Solitary

in R statistical software [33].
formation on diet and sociality of each species were obtained from the Handbook of the Mammals of the World [34]. The animals were divided into 3 categories, depending on their sociality: solitary, social, and opportunistically social (Table 2). The last category was added to account for species that form groups only as young individuals (eg, cheetahs) or only occasionally form packs for hunting (eg, black-backed jackals). For diet, carnivores were placed into 2 groups: species that commonly include birds in their diets and species that rarely or never consume birds (Table 2).

To test for effects on seroprevalence, a generalized linear model with binomially distributed errors was used. As response variables, the number of seropositive and seronegative individuals for each species were included. To test for effects on HA diversity, a linear model with normally distributed errors was used. In both models, we included diet and sociality as categorical predictors (Table 2). For post hoc analyses of significant predictors, the Tukey multiple comparison of means test, using the package "multcomp" [35] in R statistical software, was used [33].

RESULTS

Prevalence and Diversity of IAVs in African Mammals Results obtained with the influenza virus antigen–based protein microarray system [36–38] indicated that herbivores had been exposed to a more restricted range of IAV subtypes than carnivores (Figure 1). None of the 9 African elephants (Loxodonta africana) or 10 springbok (Antidorcas marsupialis) were seropositive for any of the HA

subtypes tested. The serum specimen from 1 of 10 black rhinos (Diceros bicornis) reacted very weakly to H5, with a titer just above 20, and 1 of 10 wildebeest (Connochaetes taurinus) had antibodies against H4 and H11. The percentage of herbivores with low protein microarray antibody titers detected against 3 of 16 HA proteins (H4, H5 and H11) tested was 5.12%.

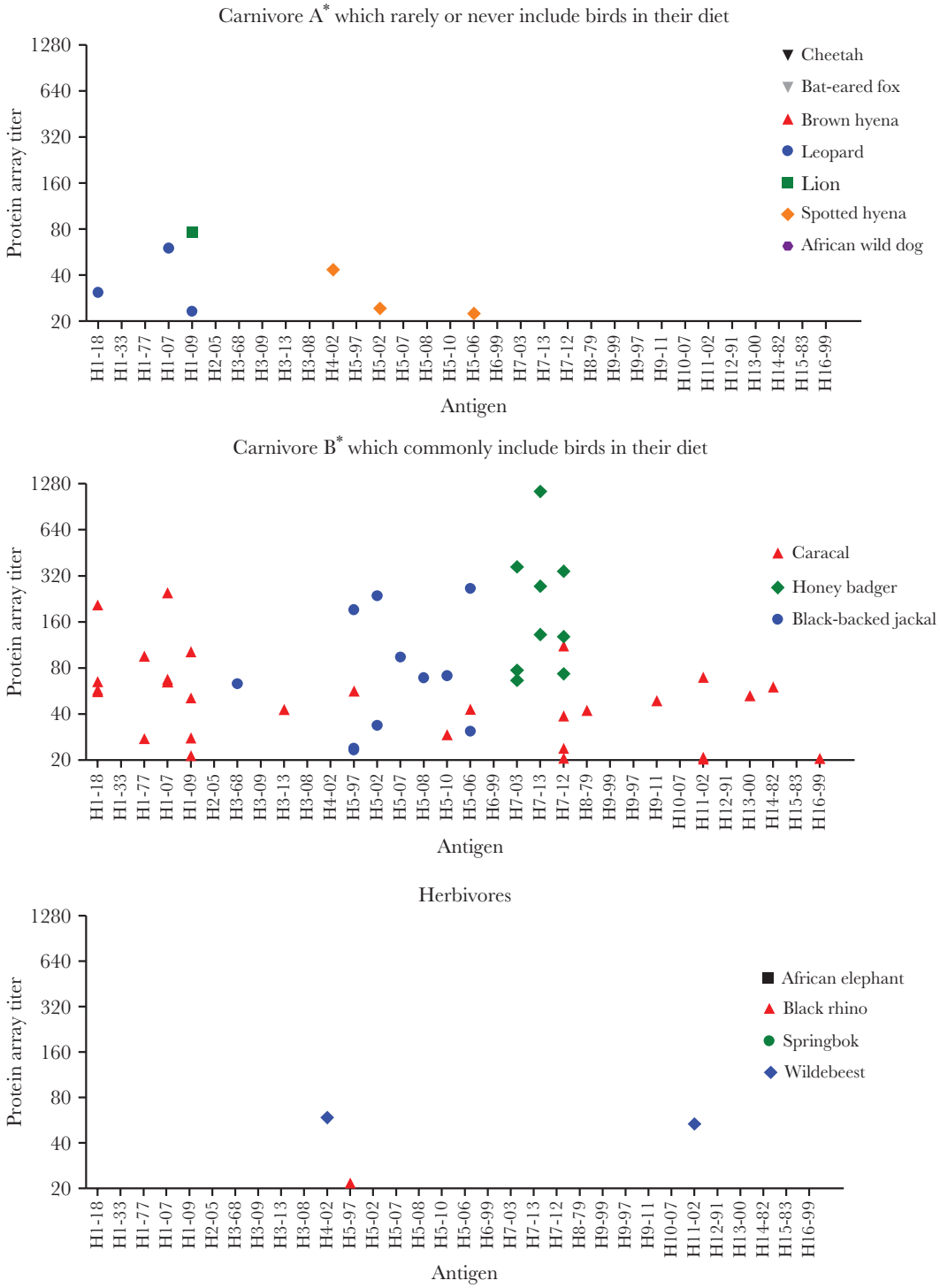

Figure 1. Overview of protein microarray titers for all hemagglutinin (HA) part antigens, by diet and species. Antigens are abbreviated as described in Supplementary Table 1. The lower limit of detection was a titer of 20; titers <20 were regarded as negative findings and are not depicted. *The carnivore A group comprises carnivores that rarely or never include birds in their diet, and the carnivore B group comprises carnivores that commonly include birds in their diet.

Carnivore sera reacted more often and with a greater diversity of HA antigens, with a seroprevalence of 20.83%, representing 11 of 16 HA proteins tested. Among the large felid species, the 4 cheetahs (Acinonyx jubatus) tested seronegative for HA-specific antibodies, while 1 of 10 lions (Panthera leo) and 1 of 9 leopards (Panthera pardus) showed reactivity to H1 antigens. Among the smaller felids, 4 of 8 caracals (Caracal caracal) had antibodies against H1, H3, H5, H7, H8, H9, H11, H13, H14, and H16 antigens. All 4 brown hyenas (H. brunnea) had negative test results, and sera from 2 of 9 spotted hyenas (Crocuta crocuta) reacted with H4 and H5 antigens (Figure 1). Among the canid species, 4 bat-eared foxes (Otocyon megalotis), 7 African wild dogs (Lycaon pictus), and 10 black-backed jackals (C. mesomelas) were screened. Four jackals show reactivity to IAV H3 and H5 antigens; all other canid species samples had negative results. Three of 7 honey badgers (M. capensis) belonging to the Mustelidae family showed reactivity to H7 antigens (Figure 1).

HI Assays

To confirm the protein microarray results, HI assays were performed with sera from lions, jackals, and honey badgers that showed reactivity in the protein microarray experiments. Comprehensive analysis could not be performed because the HI antibody assays specifically test for the subset of antibodies to the receptor-binding domain of influenza viruses, which would require a set of viruses representative of the region that is not available. Therefore, for confirmation, we selected influenza viruses isolated in other regions but for which some evidence exists of widespread circulation. An HI assay using the 2009 pandemic influenza virus strain A/California/007/09 confirmed the lion

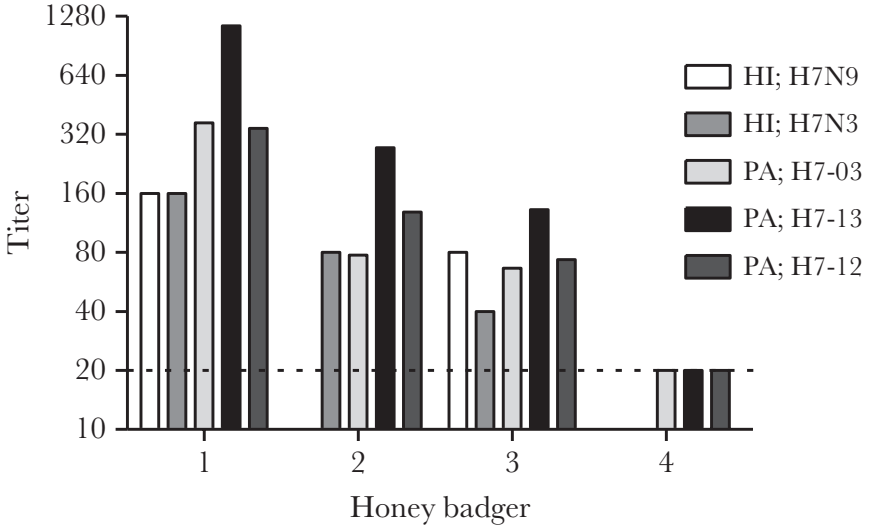

Figure 2. Hemagglutination inhibition (HI) assay titers, using 2 different influenza virus strains, and protein microarray (PA) titers, using 3 different influenza virus strains, for 4 honey badgers. Dotted line is the starting dilution for the protein microarray.

H1 microarray result, with titers of 40 and 76 for the HI assay and the protein microarray, respectively. All lions with negative results of the protein microarray experiment also had negative HI assay results. HI assay with influenza virus strains A/Anhui/001/2013(H7N7) and A/Mallard/ Neth/12/00(H7N3) confirmed the H7 reactivity observed in honey badgers (Figure 2). Jackals had negative results of HI assays, but we cannot exclude that an H5 strain not included in the HI assay (eg, a low-pathogenic H5 strain) was responsible for the production of antibodies detected by protein microarray.

Table 3. Results of Linear Models Used to Assess the Effects of Diet and Sociality on Seroprevalence and Hemagglutinin (HA) Diversity

Response, Predictor Variables	df	F	*P*
Seroprevalence			
Diet	*2*	8.81	<.001
Sociality	2	0.26	.774
HA diversity			
Diet	2	8.28	.009
Sociality	2	0.98	.413

Table 4. Results of Pairwise Post Hoc Tests to Assess the Effects of Diet Categories on Seroprevalence and Hemagglutinin (HA) Diversity

Comparison[a]	Estimate ± SE	*t*	*P*
Seroprevalence			
Carnivore B vs carnivore A	−1.99 ± 0.91	−2.18	.074
Carnivore B vs herbivore	−2.57 ± 1.05	−2.45	.038
Carnivore A vs herbivore	−0.58 ± 0.90	−0.64	.794
HA diversity			
Carnivore B vs carnivore A	−0.76 ± 0.25	−3.06	.032
Carnivore B vs herbivore	−0.79 ± 0.27	−2.89	.042
Carnivore A vs herbivore	−0.02 ± 0.19	−0.13	.991

Post hoc analyses of significant predictors were performed using the Tukey multiple comparison of means test.

Abbreviation: SE, standard error.

[a]The carnivore A group comprises carnivores that rarely or never include birds in their diet, and the carnivore B group comprises carnivores that commonly include birds in their diet.

Statistical Analysis of Seropositivity and HA Diversity

For both measures, the estimated values of Pagel's lambda [30] were <0.001, which in both cases did not significantly differ from 0 (P = 1 in both cases). Accordingly, we found no support for the prediction that more-closely related

species have a more similar IAV prevalence and HA diversity, compared with more-distantly related species.

Using generalized linear models, a statistically significant effect of diet on both measures was detected ($P < .001$ for seroprevalence, and $P = .009$ for HA diversity; Table 3). However, no significant effect of sociality was observed ($P = .77$ for seroprevalence, and $P = .41$ for HA diversity; Table 3). Therefore, there was no support for the prediction that higher levels of sociality are associated with high IAV prevalence and HA diversity. However, post hoc tests revealed that the effect of diet was mainly caused by an increased seroprevalence and HA diversity in carnivores that commonly include birds in their diet (Table 4 and Figure 3).

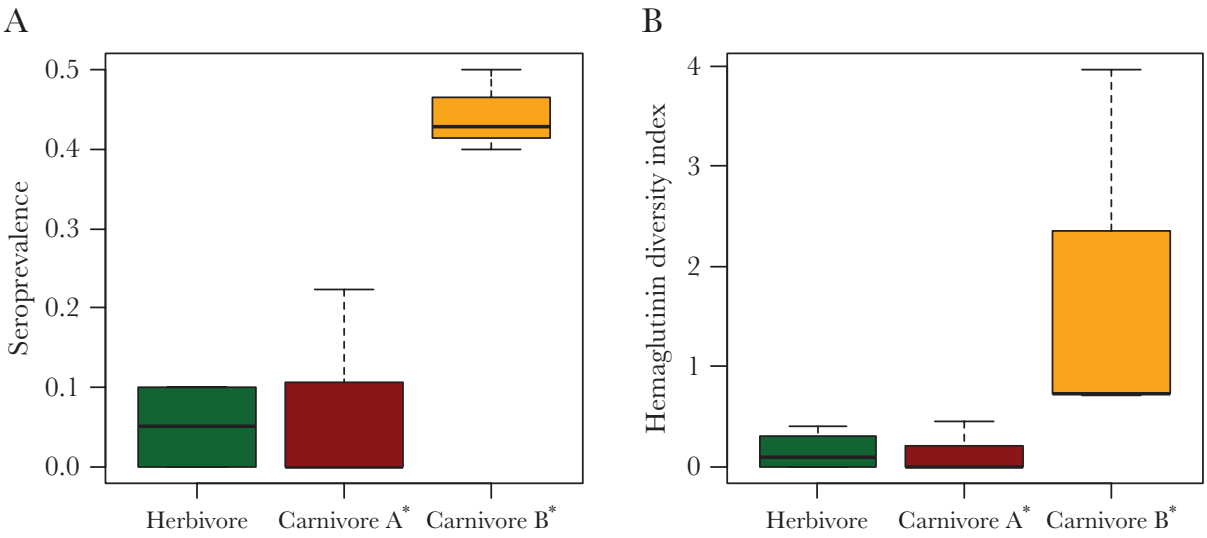

Figure 3. Box plots illustrating the relationships between diet and influenza A virus seroprevalence (A) and hemagglutinin diversity (B). To correct for variation in sample sizes among species, the hemagglutinin diversity index for each species represents the number of hemagglutinin subtypes that is expected for a sample of 4 individuals. *The carnivore A group comprises carnivores that rarely or never include birds in their diet, and the carnivore B group comprises carnivores that commonly include birds in their diet. (see Table 2 for details).

DISCUSSION

Influenza virus surveillance in wild animals focuses mainly on waterfowl and either high- or low-pathogenic H5 and H7 influenza virus subtypes. In a complex and diverse environment that hosts numerous bird and mammalian species, the potential for cross-species transmission may be greater. Standard approaches, such as HI assays, are sensitive and very specific but may not work if novel, antigenically distinct strains emerge. Detecting antibodies by using a more general method as represented by the protein microarray [36–38] may provide first-line evidence of influenza virus transmission within a complex ecological context. The seropositive species found in the current study represent the first evidence of serological exposure of different mammalian species to a diverse number of avian IAVs.

Further clarification concerning viral adaptation and exposure dynamics within the Namibian ecosystem and, furthermore, the probability of mammal-to-mammal transmission will only be possible with further sampling, organized surveillance, and viral isolation.

To our knowledge, the current study represents the first indication of IAV infection in wild African mammals (except for frugivorous bats [Eidolon helvum] in Ghana [38]). Caron et al [39] reported detection of low-pathogenic avian IAV H5 and H7 in waterfowl in Zimbabwe. This was consistent with findings by Cumming et al [3], who reported H5 and H7 IAV strains, as well as H6, H1N8, and H3N8 IAV strains, in birds in South Africa, Botswana, Zimbabwe, and Mozambique. A similar pattern was observed in South African ostriches, ducks, and wild birds, in which low-pathogenic H7N1, H5N9, H9N2, H6N8, and H10N1 IAV strains were detected [40]. The results in birds are consistent with our observations in mammals, in which H1, H5, and H7 were the most frequent IAV subtypes observed and connect the strains observed most frequently in mammals to the strains circulating in the bird population in the study area. In 2009, pandemic IAV H1N1, which emerged in the United States and Mexico, reached the human population of South Africa [41], but whether wild animals were exposed during this first introduction of the 2009 pandemic strain remains unknown.

The broad taxonomic diversity of species that can be infected by avian IAVs suggests that influenza virus may not be strictly host specific in general [15]. It is furthermore unclear whether influenza virus infection is influenced by host intrinsic resistance factors, which should correlate with phylogenetic relationships among species, or is driven by exposure to influenza virus reservoirs (eg, ecological factors such as interactions between natural hosts and susceptible animals).

We made 3 predictions of the main drivers of IAV exposure in Namibian wild mammals, 2 of which were not supported. Considering the close phylogenetic relationship among some of the tested species, one could predict that the similarity in biological barriers could influence IAV exposure. For example, some natural resistance is afforded to species with α2,6 sialic acid receptors in the upper respiratory tract, which bind avian IAVs poorly [42, 43]. However, phylogenetic relatedness was not significantly correlated with IAV exposure or with the diversity of strains identified in each species. Therefore, more-closely related biological barriers to infection do not play an apparent role in limiting IAV exposure in the current study.
In epidemiology, one of the main factors affecting disease is sociality (eg, animal density) [44]. Consequently, it could be expected that highly social animals would display higher prevalence and abundance of avian IAVs than less social or solitary mammals. However, we observed no statistically

supported association of sociality and IAV exposure or strain diversity. The lack of an effect may indicate that the observed infections represent bird-to-mammal infections and that mammal-to-mammal transmission, in which sociality would be expected to play a role in transmission, has not occurred.

Captive carnivores, including tigers, leopards, dogs, cats, and raccoons, have been observed with influenza symptoms subsequent to contaminated meat consumption [8, 11, 45–49]. Most of the cases included highly pathogenic avian IAVs, which are known to be less host specific. Highly pathogenic avian IAVs can be cleaved by a broader number of intracellular subtilisin- like proteases than low-pathogenic IAVs, and they may have decreased tissue tropism, resulting in a higher likelihood of systemic infection due to higher distribution and increased replication rates [9]. Therefore, animals consuming carcasses infected with highly pathogenic avian IAVs may be more likely to become infected, owing to decreased avian IAV host specificity. In the current study of wild mammals, a significant association was found for the prediction that a diet including birds would lead to increased IAV exposure and higher strain diversity. The results suggest that the key driver for both infection and strain exposure is direct contact with the natural avian reservoirs of IAVs. Further molecular biological work will be necessary to determine whether the strains identified serologically represent low- or highly pathogenic IAV strains.

Additional environmental factors can increase exposure to birds, such as direct or indirect contact with bird feces or contaminated water. Animals congregate at water holes during the dry seasons, where virus-contaminated surface water could potentially act as an intermediate viral vector [50]. Such environmental factors may explain the sporadic IAV exposure among herbivores and non–bird-consuming carnivores.

In summary, we found that bird-eating Namibian carnivores are more often seropositive than other mammals and may have an important but overlooked role in influenza epizootics. Further research should clarify the exact strains involved and whether viral adaptation to the host occurs that could lead to transmission among mammals and evolution of mammal- adapted influenza virus strains. The results suggest that IAVs are not only an emerging zoonotic pathogen of concern in temperate regions, but also of potential epizootic concern in savannah-steppe environments.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments.
We thank Bettina Wachter, Jorg Melzheimer, Maren de Vries, and Sonja Heinrich, for storage and import of the samples; and Katja Pohle and Karin Hoenig, for technical support.

Financial support.
This work was suppported by the Leibniz Gemeinschaft (SAW-2015-IZW-1 440 to E.-S. S., M. F., O. H. K. A., A. D. G., and G. A. C.) and the Erasmus Medical Center Department of Viroscience.

Potential conflicts of interest.
All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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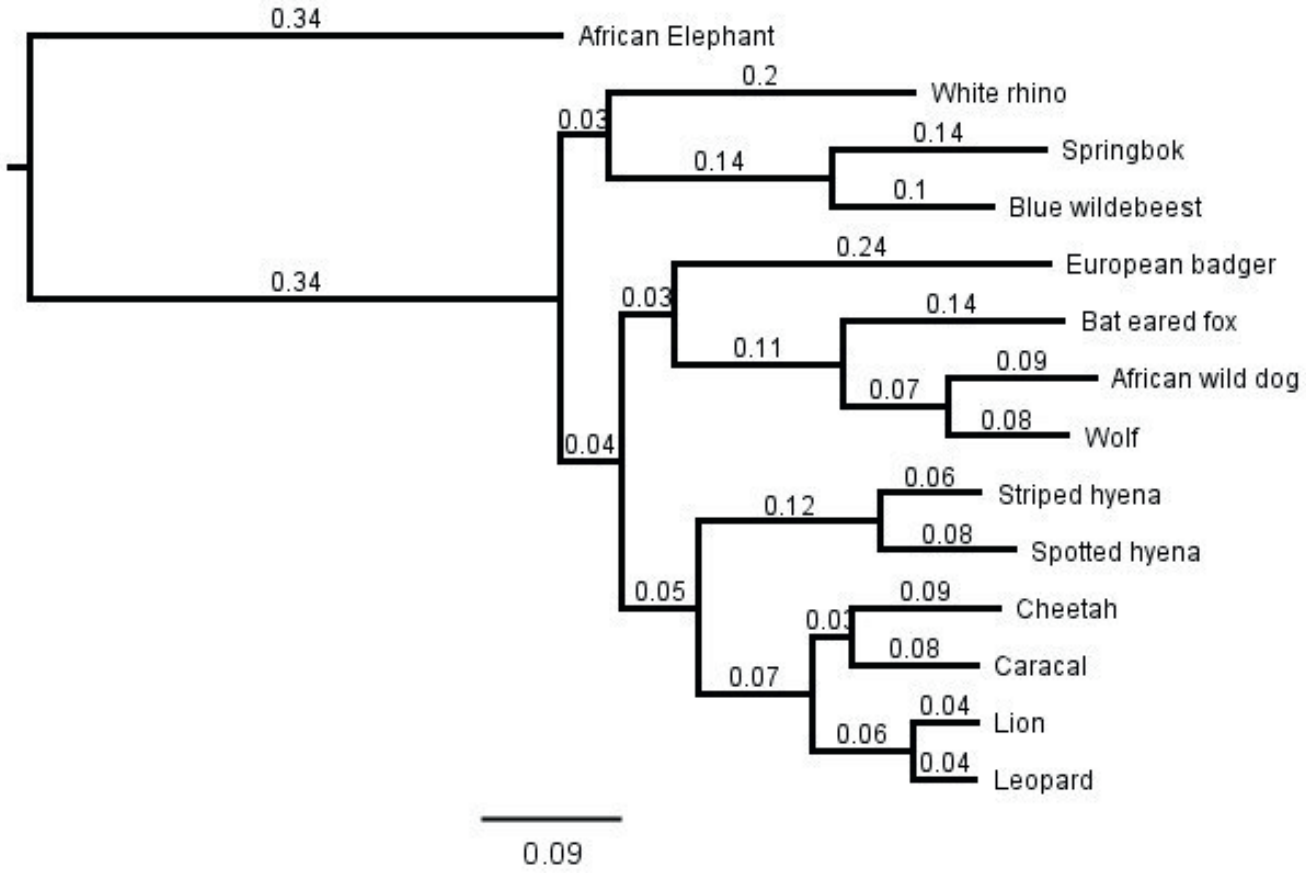
Supplementary material

Supplementary Table 1. Recombinant hemagglutinin (HA)-proteins included in protein microarray

CODE	SUBTYPE	STRAIN
H1-18	H1N1	A/South Carolina/1/18
H1-33	H1N1	A/WS/33
H1-77	H1N1	A/USSR/92/1977
H1-07	H1N1	A/Brisbane/59/2007
H1-09	H1N1	A/California/6/2009
H2-05	H2N2	A/Canada/720/05
H3-68	H3N2	A/Aichi/2/1968
H3-09	H3N9	A/VICTORIA/210/2009
H3-13	H3N2	A/Switzerland/9715293/2013
H3-08	H3N8	A/equine/Gansu/7/2008
H4-02	H4N6	A/mallard/Ohio/657/2002
H5-97	H5N1	A/Hong Kong/156/97 (clade 0)
H5-02	H5N8	A/duck/NY/191255-59/2002 low pathogenic
H5-07	H5N3	A/duck/Hokkaido/167/2007 low pathogenic
H5-08	H5N1	A/chicken/Egypt/0879-NLQP/2008 (clade 2.2.1.1)
H5-10	H5N1	A/Hubei/1/2010 (clade 2.3.2.1)
H5-06	H5N1	A/Turkey/15/2006 (clade 2.2)
H6-99	H6N1	A/quail/HK/1721-30/99
H7-03	H7N7	A/Chicken/Netherlands/1/03
H7-13	H7N9	A/chicken/Anhui/1/2013
H7-12	H7N3	A/chicken/Jalisco/CPA1/2012
H8-79	H8N4	A/pintail duck/Alberta/114/1979
H9-99	H9N2	A/Guinea fowl/Hong Kong/WF10/99
H9-97	H9N2	A/chicken/Hong Kong/G9/97 (G9 lineage)
H9-11	H9N2	A/Chicken/India/IVRI-0011/2011
H10-07	H10N7	A/blue-winged teal/Louisiana/Sg00073/07
H11-02	H11N2	A/duck/Yangzhou/906/2002
H12-91	H12N5	A/green-winged teal/ALB/199/1991
H13-00	H13N8	A/black-headed gull/Netherlands/1/00
H14-82	H14N5	A/mallard/Astrakhan/263/1982
H15-83	H15N8	A/duck/AUS/341/1983
H16-99	H16N3	A/black-headed gull/Sweden/5/99

Supplementary Figure 1. Maximum likelihood tree of the 14 host species, constructed based on a MUSCLE alignment of mitochondrial coding regions. Sequences of African elephant (*Loxodonta Africana,* NC_000934.1), bat eared fox (*Otocyon megalotis,* KY776502.1), wolf (*Canis lupus campestris,* KC896375.1), caracal (*Caracal caracal,* NC_028306.1), cheetah (*Acinonyx jubatus* AY463959.1), European badger (*Meles meles*, NC_011125.1), leopard (*Panthera pardus,* KP001507.1), lion (*Panthera leo,* KP001496.1), white rhino (*Diceros bicornis,* FJ905814.1), spotted hyena (*Crocuta crocuta,* NC_020670.1), springbok (*Antidorcas marsupialis,* NC_020678.1), African wild dog (*Lycaon pictus,* KT598692.1), striped hyena (*Hyaena hyaena*, JF894376.1) and wildebeest (*Connochaetes taurinus,* JN632627.1) were retrieved from the NCBI database. Branch lengths are proportional to evolutionary distance



Chapter 3

Bearing the brunt: Mongolian khulan (Equus hemionus hemionus) are exposed to multiple influenza A strains

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Running Title: Influenza A exposure in Mongolian khulans
Keywords: Influenza A, virus, serology, Asiatic Wild Asses, exposure, antibodies
Word count abstract: 193
Word count text: 2418

Abstract. The majority of influenza A virus strains are hosted in nature by avian species in the orders of Anseriformes and Charadriformes. A minority of strains have been able to cross species boundaries and establish themselves in novel non-avian hosts. Influenza viruses of horses, donkeys, and mules represent such successful events of avian to mammal influenza virus adaptation. Mongolia has over 3 million domestic horses and is home to two wild equids, the Asiatic wild ass or khulan (*Equus hemionus hemionus*), and Przewalski's horse (*Equus ferus przewalskii)*. Domestic and wild equids are sympatric across most of their range in Mongolia. Epizootic influenza A virus outbreaks among Mongolian domestic horses have been frequently recorded. However, the exposure, circulation and relation to domestic horse influenza A virus outbreaks among wild equids is unknown. We evaluated serum samples of Asiatic wild asses in Mongolia for antibodies against influenza A viruses, using modified protein microarray technique. We detected antibodies against hemagglutinin (H) H1, H3, H5, H7, H8 and H10 influenza A viruses. Asiatic wild asses may represent a previously unidentified influenza A virus reservoir in an ecosystem shared with populations of domestic horses in which influenza strains circulate.

Importance. Influenza A virus monitoring of domestic animals is often undertaken independent of the ecological context in which the animals exist. Domestic horses in Mongolia are sympatric and have largely unconstrained contact with wild equids within the Mongolian steppe ecosystem. Our results suggest that the Asiatic wild asses are exposed to known equine influenza A strains H3N8 and H7N7 and additional influenza strains not previously described in horses. H7N7 has been considered to be absent from the region but is clearly not. Asiatic wild asses may therefore represent an influenza A reservoir of relevance to the domestic and wild equid population of eastern Asia.

Introduction

Historically, two major strains of Equine Influenza virus (EIV) have caused influenza virus outbreaks in domestic equids. The first identified EIV, influenza A/H7N7 or equine-1, was isolated from horses in 1956 [1]. Influenza A/H3N8 or equine-2 was subsequently reported and remains the major cause of equine influenza [2]. While H7N7 EIV is thought to be equine-specific with limited but unique variation in the HA gene [3], H3N8 EIV appear to bind to avian-like receptors in the upper respiratory tract of horses suggesting a recent avian origin of the strain[4]. Moreover, previous H3N8 influenza virus outbreaks in dogs [5], their isolation from a Bactrian camel in Mongolia [6], and some evidence for human infection [7], indicate that horses are not the only host for H3N8 viruses, and that their zoonotic potential might be underappreciated. Mongolia, with a current population of domestic horses exceeding 3 million, has suffered several EIV outbreaks [8]. The first two outbreaks, 1974-75 and 1983-84, were caused by H7N7 EIV and the last three, 1993-94, 2007-08 [9] and 2011 were caused by H3N8 EIV. After 1984, H7N7 EIV was not isolated and is considered extinct in the region.

In addition to domestic horses, Mongolia is home to the Przewalski's horse (*Equus ferus przewalskii)*, and hosts the biggest population of Asiatic wild ass (or khulan, *Equus hemionus hemionus*) in Central Asia [10]. The distribution of khulan overlaps with other free-living ungulate species, such as goitered gazelles (*Gazella subgutturosa*), Mongolian gazelles (*Procapra gutturosa*), and free ranging Bactrian camels (*Camelus ferus*). Most importantly their distribution overlaps with local livestock including domestic horses which outnumber wild ungulates by several orders of magnitude. Disease transmission between domestic and free-living populations is possible through sharing pasture and waterholes. EIV outbreak dynamics in wild equids from Central Asia are poorly understood. In 2007 an H3N8 influenza (A/equine/Xinjiang/4/2007) outbreak was reported in a Przewalski's horse population in the Chinese part of the Gobi with a 5% mortality rate [11]. Influenza exposure in khulans, however, remains uncharacterized. Mongolia also has a high diversity of wild birds, including migratory waterbirds, that use Mongolia as a stop-over during their annual migrations. The Central and the East Asian flyways passing through Mongolia are critical to influenza ecology (Figure 1). Therefore, we sought to investigate the exposure to influenza viruses in Mongolian khulan, as a first step in understanding their role in the ecology of equine influenza.

Materials and Methods

The study took place in the Southern Gobi Desert in Mongolia, and was approved by the ethical committee of the University of Veterinary Science in Vienna (ETK-15/03/2016) and the Mongolian Government (05/5656). Twen-

ty-one adult khulan (8 stallions and 13 mares) were anesthetized and nasal swabs, serum and blood samples collected. The method of choice for efficient chemical capture in the Gobi desert is from a moving jeep. After successful detection of khulan in the steppe, and initiation of the chase, there is a cut off time of 15 min for animal welfare reasons which determines when capturing will end. Subsequently, a new khulan group needs to be found before continuing. The time to capture (from detection and initiation of the chase to reversal of the anesthesia to being ready to resume the search for a new animal) for individual animals ranged from approximately 1 hour to several days.

The animal sampling expedition was part of a radio collaring project, in which habitat fragmentation, due to new mining-related infrastructures in Southern Gobi was investigated. Khulans were captured in two different locations, one near the mining-infrastructure site and one near the Ergeliin Zoo protected area (Figure 1). All animals were darted from a moving jeep, using a Daninject JM CO2 darting gun [12]. None of the khulan demonstrated clinical symptoms of EIV or other infectious diseases when handled. Samples were stored immediately at -20°C in a portable freezer in Mongolia, transported on dry ice to Austria in full compliance with the Convention on International Trade in Endangered Species (CITES) and stored at −80 °C until laboratory analysis at the Research Institute of Wildlife Ecology, University of Veterinary Medicine, Vienna. Due to field conditions and the absence of a mobile laboratory, blood samples could not be processed on site, so that only 13 of 21 serum samples could be used and all were severely hemolysed.

A protein microarray (PA) technique as described previously [13,14,15,16],was used to identify the influenza virus strains in the khulan serum samples. Samples where inactivated in a water bath at 56° C for 4 hours due to regulations for testing of animal samples from food and mouth disease endemic regions. Serum samples from 3 kulans were tested against different secondary antibodies in order to determinate the highest sensitivity; protein A, protein G and anti-horse. Anti-horse IgG showed highest overall response.

Briefly, thirty-two recombinant proteins of different influenza A virus antigens were printed onto 16-pad nitrocellulose Film-slides (Oncyte avid, Grace Biolabs, Bend, OR, USA). All presently known influenza A virus HA-types are represented on the array (Table 1), except for hemagglutinin type 17 and 18, as those are only detected in bats. Slides were treated with Blotto-blocking buffer to avoid non-specific binding (Thermo Fischer Scientific Inc., Rockford, MA, USA) for 1 hour at 37°C in a moist chamber. After washing the slides were incubated with a fourfold dilution series of the khulan serum starting from 1:20 to 1:1280. After 1 hour incubation at 37 °C, slides were washed and incubated with a 1:500 dilution of the anti-horse IgG conjugated to Alexafluor 647 (Jackson immunoresearch). A last washing step was done to remove unbound conjugate, after which the slides were dried and scanned using a Powerscanner (Tecan). Spot intensities were determined, and titer heights

were calculated by curve fitting using R (R Statistical Computing, version 3.1.0, Vienna, Austria). Titers less than 20 were set to 20.
Protein microarray results were confirmed depending on the availability of strains with a probability of being related to the field strains. Investigation of viral shedding was attempted from nasal swabs (see Supplementary material); however, as none of the animals were actively shedding virus, genome sequences were unavailable introducing uncertainty as to which strains to use in confirmatory serological assays. Hemagglutination inhibition assays (HI) (see Supplementary material), Virus Neutralization Test (VNT) (Supplementary material) and Single Radial Hemolysis Assay (SRH) were used to confirm the results of PA on a subset of samples.
Seven khulan serum samples (4 microarray positive and 3 negative as controls) were tested against strains H3N8 (A/equine/Richmond/1/07), H7N7 (A/Equine Praque/1/56), and H7N3 (A/Mallard/Netherlands/12/00) using SRH according to the OIE recommendations [17]. Positive and negative reference anti-equine influenza serum were used in each plate as controls. Fresh sheep erythrocytes (obtained from Berlin zoo) and/or chicken erythrocytes (Labor Dr. Merk, Germany) were washed and prepared to a final concentration of 8% in saline/HEPES buffer. Erythrocytes were sensitized with each virus independently, mixed with guinea pig complement (Sigma) and 1% agarose gel, and finally spread on a plate. Holes were punched in the gel and 10 µl of heat inactivated (56°C for 30 min) serum, including positive and negative controls, were added to each well and plates were incubated at 34°C for 20 hours in a humid box. The area of hemolysis was measured and expressed in mm^2. The assay was repeated two times independently.

Results

Viral detection was attempted from nasal swabs using qPCR but no virus could be detected. The result is not surprising as 460 domestic free-ranging Bactrian camels were similarly screened yielding a single influenza A virus positive individual [6]. Considering none of the animals displayed clinical symptoms of infection, the lack of actively shedding individuals is consistent with expectations.
To detect exposure to influenza in non-shedding individuals, a protein microarray (PA) technique testing 32 hemagglutinin recombinant proteins (HA1-part) from type H1 to H16, as described previously [13,14,15,16], was used to profile the antibodies to influenza viruses in the khulan serum (Table 1). Six animals were negative, whereas 7 animals had reactivity detectable by microarray to one or more antigens. These were low levels of reactivity to H5 (2 animals), H8 and H10 antigen (1 animal each), and low to moderate titers against H1 (1 animal), and H7 (2 animals). Five khulans showed reactivity to H3-08, which is the horse influenza strain known to circulate in Mongolia. This reactivity was specific for the EIV H3 antigen, other antigens (represent-

ing strains isolated from humans) were negative (Figure 2). The two khulan serum samples which reacted with H7 antigen, reacted specifically to the Dutch H7N7 strain (H7-03, A/Chicken/Netherlands/1/03), but not to the Chinese H7N9

Confirmation of the PA results using hemagglutination inhibition assays was not possible because the serum was severely hemolysed and agglutination was detected in the control well without virus. VNT was tested on a subset of serum samples (Table 2) using one H3N8 equine, and two H7 strains (see supplemental information). Although minimal replicates were used, VNT did confirm the H3N8 result of the PA, but failed to confirm the H7 result (Table 2). Single radial hemolysis (SRH) was performed using A/equine/Richmond/1/07, A/Equine Praque/1/56 and A/Mallard/Netherlands/12/00. Two of four H3 positive khulan in the protein microarray reacted with A/equine/Richmond/1/07 with a hemolysis area of 35 and 60 mm^2. The two samples that could not be confirmed had lower antibody titer as determined by the protein microarray and were likely below the detection limit of the SRH assay (Table 2). The H7 positive khulan in the protein microarray, reacted with both A/Equine Praque/1/56 and A/Mallard/Netherlands/12/00; the hemolysis area was 35 mm^2. The hemolysis areas of positive and negative control serum were 148 and 12 mm^2, respectively.

Discussion

Although we could not detect viral genomes to further define the strains circulating among wild equids, our serological results suggest that equids may be exposed to more influenza viruses than previously considered. Virus detection is often limited by the short window in which the virus is present, and therefore screening for antibodies, which often persist longer than the virus itself, provides information about past infections and virus diversity in animal populations [15]. While sampling of twenty-one individual animals may seem low, one has to keep in mind that khulans are extremely skittish animals, and normally flee human presence even when separated by several kilometers distance. Anesthesia and sampling of non-domestic equids, particularly under the physically challenging and remote environment of the Gobi Desert, can be difficult, for both animals and humans, and not always successful [12]. Furthermore, khulans are a red list species globally and nationally and capture permits are granted only after careful evaluation of the risks and benefits. On these grounds, capture permits for the mere sampling of an endangered species without an imminent need have little chance of approval.

The difficult terrain, with dry river beds, low mountains, bushes, shrubs and desert basins, severely restricts successful outcomes. Capture (from detection and initiation of the chase to reversal of the anesthesia to being ready to resume the search for a new animal) for one individual takes approximately 1 hour under the best conditions but ranges to several days, if khulans are not

found in the vast Gobi ecosystem [18]. In our study, the number of animals captured exceeded the expectations for our short 2-week window. These challenges need to be taken into account when evaluating this study.

Our findings suggest that khulans might be susceptible to more influenza A viruses than previous thought. The broad number of influenza strains is consistent with the widespread SAα2,3-Gal and Saα2,6-Gal expression in the horse upper respiratory tract 19, suggesting that khulans could potentially be infected by both mammalian and avian influenza viruses.

The most commonly detected antibodies were against H3 EIV HA1 antigens, consistent with data on low vaccination rates and therefore an ongoing circulation of these viruses among Mongolian horses[8,9]. We found evidence for exposure to influenza viruses with a hemagglutinin of subtype H7. H7N7 equine influenza is considered extinct in the region, although some studies still report serological evidence of the strain [20]. The protein microarray and confirmatory SRH reacted both with H7N7 A/Equine Praque/1/56 and H7N3 A/Mallard/Netherlands/12/00. SRH has been demonstrated to be the most sensitive serological assay for equine influenza viruses [21], and co-circulation of both subtypes cannot be excluded. Other H7 subtypes circulate in wild birds in Southeast Asia and viral isolation would further clarify to which specific H7 influenza virus khulan might be exposed in Mongolia. Positive khulans against H1 strains may have been infected during an H1N1 pandemic in 2009 [22]. Sajid et al. [23] reported similar results in horses in Pakistan during an EIV outbreak. Two khulans had antibodies against H5 influenza viruses. Similar results have been reported for donkeys, a domestic equid, suggesting equids are susceptible to highly pathogenic H5N1 influenza strains [24].

Individual animals were positive for HAs of influenza A H8 and H10, suggesting that sporadic infections with viruses belonging to these subtypes have occurred. However, we cannot exclude that multiple known or unknown strains of H1, H5, H8 and H10 cross reacted in the microarray assay due to the haemolytic nature of the khulan serum or that the viral strains eliciting the immune response are divergent from known strains. The lack of knowledge of strains circulating in wild animal populations constrains assay confirmation and represents a potential area where further research would be beneficial.

A possibility is that these viruses co-circulate with H3N8 among equids in Central Asia, but occasional introduction from exposure to wild birds, other mammals or their droppings is a possible alternative [25]. In a harsh steppe-desert ecosystem such as the Gobi Desert, water can be scarce resulting in diverse species congregation at waterholes which may increase disease transmission. The upper respiratory tract of the horse express sialic acid 2,3-linked receptors, which are similar to those in wild aquatic birds. Because of this similarity in avian and equine respiratory biology [26], it is possible that equids are susceptible to a broader spectrum of influenza viruses than other mammals. Furthermore, the presence and free movement of the domestic

and wild species may be risk factors associated with the influenza exposure and transmission.
Although susceptibility of wild equids to new influenza strains may not pose a threat to their conservation status, it might represent an overlooked ecological niche for influenza virus and an alternative route of infection for other wild and domestic animals. Further epidemiological investigation of wild equids from Central Asia should clarify the diversity of influenza virus strains that infect wild equids and help to establish the monitoring of influenza virus transmission between wild and domestic equids in the area.

Authors and contributors
AG, CW conceptualized the study. AG, CW supervised the study. SES, BB, PK, CW conducted the investigation on capturing and sampling the animals. SES, EdB, KE, WA, NO, GC analyzed the data. SES, GC and ADG wrote the original draft. All co-authors contributed to the writing, review and edited of the manuscript.
Conflicts of interest
The authors declare that there are no conflicts of interest
Funding information
ESS, NO, GAC, CW and ADG were funded by a grant from the Leibniz Gemeinschaft (SAW-2015-IZW-1 440).

Acknowledgments
We acknowledge the FUW-Advanced Design Studio for their insightful suggestions for the map illustration. We thank the Ministry of Nature, Environment and Tourism of Mongolia, Dashzeveg Tserendeleg, Otgonsuren Avirmed, Enkhtuvshin Shiilegdamba (WCS), Nyamdorj Barnuud (SEA), Dennis Hosack, and Purevsuren Tsolmonjav (OT) for the logistical and practical support during khulan capture.

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Figure legends

Figure 1: Relation between khulan distribution, protected areas, sampling sites and the major migratory flyways in Mongolia. The arrows represent the outside border of each migratory flyway.

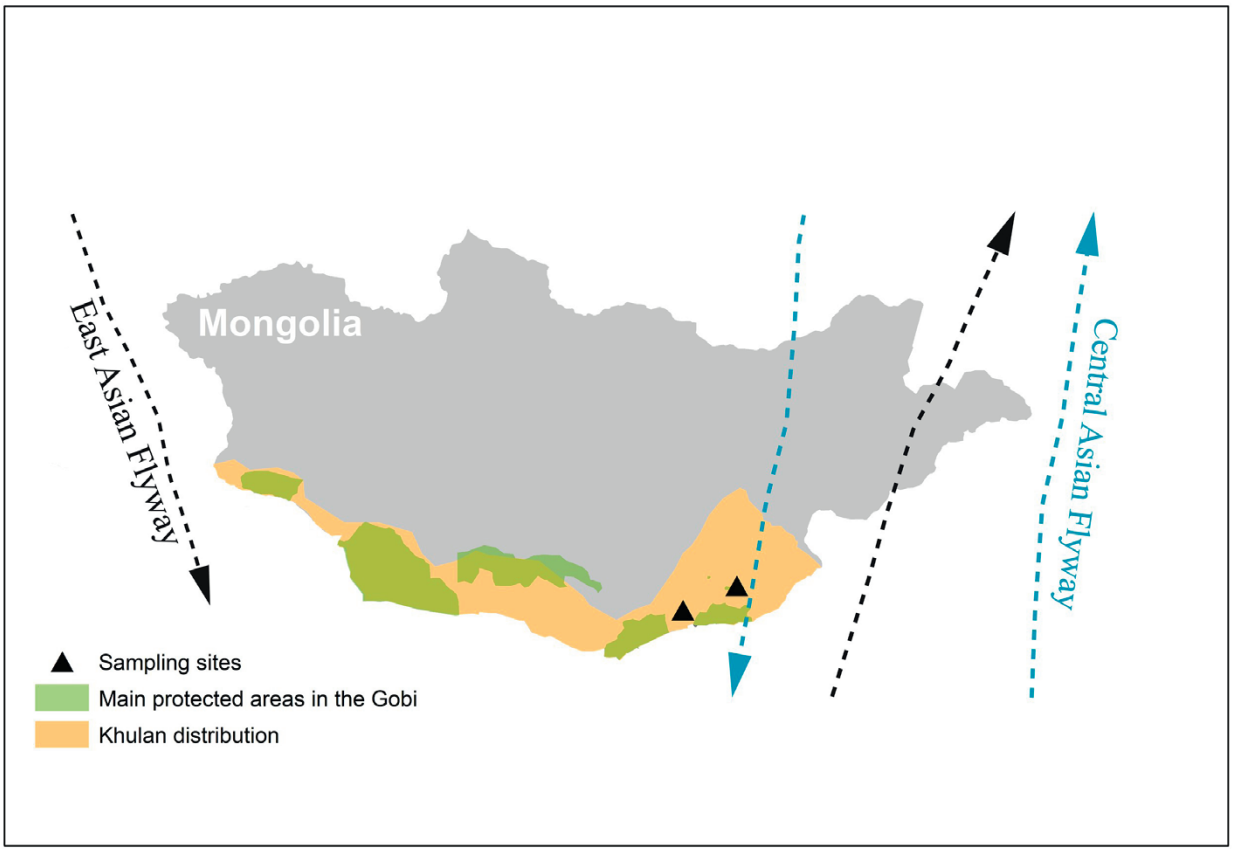

Figure 2: Antibody profiles in sera from khulans, expressed as titers (Y axis) of IgG reactivity to a range of influenza A HA1 antigens (X axis). Animal number corresponding to ID's: 1: 19742; 2:19850; 3:19845; 4:19842; 5:19852; 6:19555; 7:19848

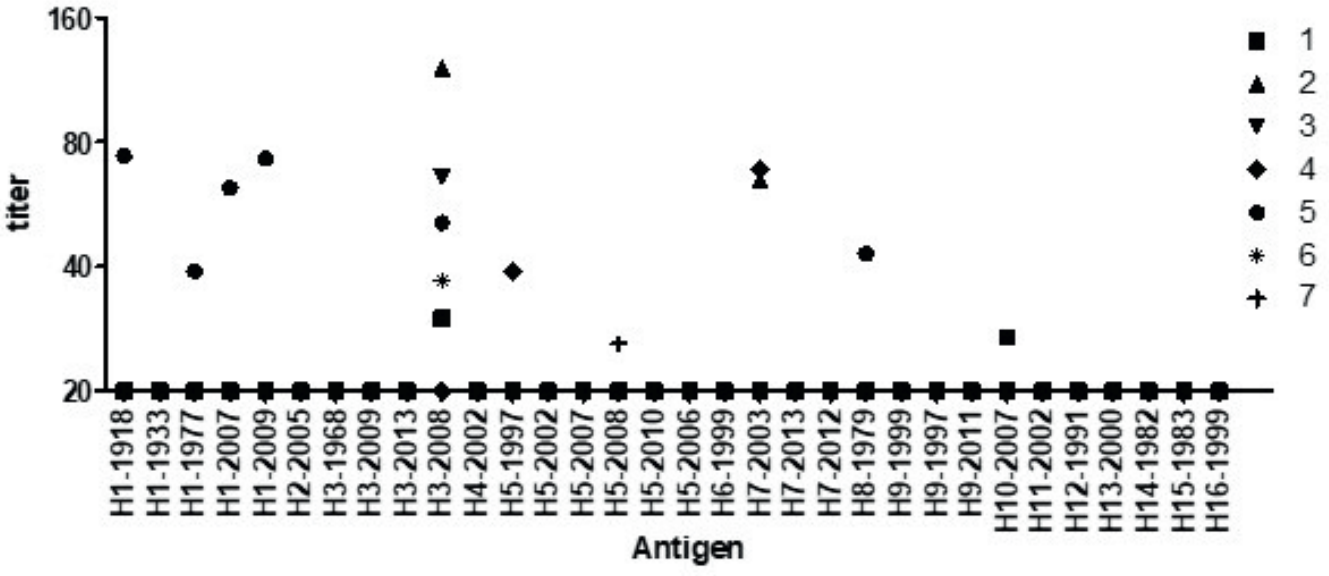

Table 1. Recombinant HA1-proteins included in the protein microarray.

CODE	SUBTYPE	STRAIN
H1-1918	H1N1	A/South Carolina/1/18
H1-1933	H1N1	A/WS/33
H1-1977	H1N1	A/USSR/92/1977
H1-2007	H1N1	A/Brisbane/59/2007
H1-2009	H1N1	A/California/6/2009
H2-2005	H2N2	A/Canada/720/05
H3-1968	H3N2	A/Aichi/2/1968(H3N2)
H3-2009	H3N9	A/VICTORIA/210/2009
H3-2013	H3N2	A/Switzerland/9715293/2013
H3-2008	H3N8	A/equine/Gansu/7/2008
H4-2002	H4N6	A/mallard/Ohio/657/2002
H5-2997	H5N1	A/Hong Kong/156/97
H5-2002	H5N8	A/duck/NY/191255-59/2002(H5N8) LP
H5-2007	H5N3	A/duck/Hokkaido/167/2007
H5-2008	H5N1	A/chicken/Egypt/0879-NLQP/2008
H5-2010	H5N1	A/Hubei/1/2010
H5-2006	H5N1	A/Turkey/15/2006 (clade 2.2)
H6-1999	H6N1	A/quail/HK/1721-30/99
H7-2003	H7N7	A/Chicken/Netherlands/1/03
H7-2013	H7N9	A/chicken/Anhui/1/2013
H7-2012	H7N3	A/chicken/Jalisco/CPA1/2012
H8-1979	H8N4	A/pintail duck/Alberta/114/1979
H9-1999	H9N2	A/Guinea fowl/Hong Kong/WF10/99
H9-1997	H9N2	A/chicken/Hong Kong/G9/97 (G9 lineage)
H9-2011	H9N2	HA1 (H9N2) A/Chicken/India/IVRI-0011/2011
H10-2007	H10N7	A/blue-winged teal/Louisiana/Sg00073/07
H11-2002	H11N2	A/duck/Yangzhou/906/2002
H12-1991	H12N5	A/green-winged teal/ALB/199/1991
H13-2000	H13N8	A/black-headed gull/Netherlands/1/00
H14-1982	H14N5	A/mallard/Astrakhan/263/1982new
H15-1983	H15N8	A/duck/AUS/341/1983
H16-1999	H16N3	A/black-headed gull/Sweden/5/99

Table 2. Results of the protein microarray (PA), virus neutralization test (VNT) and single radial hemolysis assay (SRH) against Equine Influenza virus strains. Animals marked with NT were not tested.

Animal ID	PA H3N8	PA H7	VNT H3N8	VNT H7N7	VNT H7N3	SRH H3N8	SRH H7N7	SRH H7N3
19850	121	65	16	<5	<5	Positive	Positive	Positive
19845	66	20	8	NT	NT	Positive	Negative	Negative
19852	51	20	NT	<5	<5	NT	NT	NT
19555	37	20	8	NT	NT	Negative	Negative	Negative
19742	30	20	NT	NT	NT	Negative	Negative	Negative
19848	20	20	<4	NT	NT	Negative	Negative	Negative
19842	20	69	NT	<5	<5	NT	NT	NT
19840	20	20	NT	<5	<5	NT	NT	NT
19836	20	20	NT	NT	NT	Negative	Negative	Negative
19841	20	20	NT	NT	NT	Negative	Negative	Negative
19843	20	20	NT	NT	NT	NT	NT	NT
19849	20	20	NT	NT	NT	-NT	NT	NT
13155	20	20	NT	NT	NT	NT	NT	NT

Supplementary material

Quantitative PCR (qPCR)

Two hundred µl transport medium from the swabs were extracted using the RTP DNA-RNA virus mini kit (Stratec Molecular GmbH, Germany). The RNA was reverse transcribed cDNA using an influenza specific primer followed by PCR amplification. All the reactions were performed in a single tube, by using the SensiFast™ Probe Lo-ROX One-Step kit (Bioline). Negative and positive controls were included in all experiments. Reactions were carried out in 96-well plates.

Influenza M genes primers and probes used were as follows: Primer INF FW 5'- AGA TGA GYC TTC TAA CCG AGG TCG -3', Primer INF RV 5'- TGC AAA NAC ATC YTC AAG TCT CTG -3' , Probe INF 6 FAM- TCA GGC CCC CTC AAA GCC GA –TAMRA. qPCR was performed using an ABI 7500 machine, p with the cycling conditions: 10 min at 48°C (RT reaction), 2 min at 95°C, 40 cycles of 3 sec at 95°C and 30sec at 60°C and held for 1 min at 60°C for the data collection.

Hemagglutination inhibition assays

All 13 khulan serum samples were tested for antibodies against strains A/Eq/Prague/001/1956 and A/Eq/Kentucky/001/1981 using a hemagglutination inhibition assay as previously described [2]. Serum samples were pre-treated with receptor destroying enzyme during a 16-hour incubation at 37°C, followed by 1 hour at 56 °C. Two-fold dilution series starting from 1:20 were made of the pre-treated sera and 4 hemagglutinin units of virus was added to a final volume of 75 microliter. After 30 minutes at 37°C, 25 µl of 1% turkey erythrocyte solution was added and incubated for 1 hour at 4°C. After 1 hour, hemagglutination patterns were read, and the hemagglutination inhibition titer was expressed as the highest serum dilution that still completely inhibited hemagglutination. Serum samples negative for hemagglutination inhibition in the first dilution were expressed as less than 10.

Virus neutralization test

The virus neutralization assay (VNT) was performed as described before [1], using titrated stocks of A/Mallard/NL/12/00 (H7N7, a LPAI precursor virus of the HPAI virus (H7-2003) used on the protein microarray) and A/Equine/Praque/1/54 (Equine H7). In brief, 60 µl heat-inactivated serum samples were 2-fold diluted starting with 1:10 dilution and 60 µl of the respective virus culture containing 100 $TCID_{50}$ was added to each sample. After 2 hours incubation at 37°C, 100 µl of each virus-mixture was added to a 96-well plate containing 80-95% confluent MDCK-cell culture and was incubated in 5% CO_2 environment for 2 hours at 37°C . After incubation the

virus-serum mixture was removed and cells were washed once using 200 µl virus infection medium. After washing 200 µl virus infection medium was added again and the culture was incubated in 5% CO_2 environment at 37°C. Seven days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Positive and negative controls, virus back titration and serum only controls were included in each experiment. The VNT titer was expressed as a reciprocal of the last serum dilution at which virus replication was completely blocked.

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Chapter 4

VERY LOW INFLUENZA A VIRUS PREVALENCE IN CERVIDS IN GERMAN NATIONAL PARKS

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Source: Journal of Zoo and Wildlife Medicine, 49(1):252-254.
Published By: American Association of Zoo Veterinarians
https://doi.org/10.1638/2017-0095R.1
URL: http://www.bioone.org/doi/full/10.1638/2017-0095R.1

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Abstract. Influenza A viruses are one of the most important and most studied pathogens in humans and
domestic animals but little is known about viral prevalence in non-avian wildlife. Serum samples from three freeranging
cervid species (red [Cervus elaphus], fallow [Dama dama], and roe deer [Capreolus capreolus]) were collected
from six German national parks between 2000 and 2002. The serum was tested for the presence of influenza A
antibodies using a commercial competitive enzyme-linked immunosorbent assay. Only one of 137 samples tested
positive.

Key words. Antibodies, cervids, ELISA, influenza A, serology, virus.

BRIEF COMMUNICATION

Influenza A viruses (IAV) are of major human and veterinary medical concern, causing disease in a wide range of bird and mammal species. Although of avian origin, cross-species transmission of some strains of IAV are frequent and host shifts to mammals are a major focus of research and monitoring. Viral strains such as H1N1 and H3N1 have multiple hosts and therefore mammals cannot be excluded as potential hosts in which adaptation and, in some cases, reassortment of viruses can occur and lead to the emergence of influenza epidemics.

The evidence for IAV circulation among domestic and wild animals has accumulated, though few studies have addressed non-avian wildlife influenza prevalence.[6,8] Among the Artiodactyla, apart from domestic and wild pigs,

Table 1. Number of samples ($n = 137$) of the three deer species from different German national parks.

National park	Animal samples		
	Roe deer	Red deer	Fallow deer
Sächsische Schweiz	1	9	—
Harz/Niedersachsen	16	34	—
Hochharz	—	10	—
Jasmund	—	—	32
Bayrische Wald	5	6	—
Müritz National Park	7	6	11
Total numbers	29	65	43

there have only been sporadic reports of influenza infection or exposure, e.g., Bactrian camels (Camelus bactrianus), fallow deer (Dama dama), roe deer (Capreolus capreolus), and red deer (Cervus elaphus).[2,10,12] However, a systematic survey for any wild artiodactyl has yet to be conducted. Considering many cervid species are highly abundant, sympatric with bird species carrying IAV (e.g., migrating waterfowl), and have contact with many human and domestic animal populations, characterizing IAV prevalence would determine the risk of emergence of these viruses from deer populations.

One-hundred-sixty-four blood and serum samples were collected from six national parks in Germany during the hunting seasons between 2000 and 2002 (Table 1), as part of a project examining the role of wildlife as potential reservoirs for diseases.3 The serum samples were stored at -20℃. The animals were screened for antibodies against nine different pathogens, including bovine herpesvirus-1 and bovine diarrhea virus.3 IAV was not included in the serological screening. In the current study, 137 serum samples of roe deer (n = 29), red deer (n = 65), and fallow deer (n = 43) were screened using the ID Screent Influenza A Antibody Competition Multi- Species Enzyme-Linked Immunosorbent Assay (ELISA) kit (FLUACA-10P, Nr.: FLI-B 438, IDvet Innovative Diagnostics, Grabels, 34790, France). The ELISA was performed according to the manufacturer's instructions, using 10 ll of serum in microplates that were coated with antigen A. Both positive (influenza A/N1 freezedried serum) and negative (specific-pathogen-free negative freeze-dried serum) controls were provided in the kit and were included during the screening. All samples were run in duplicate. Working with a competitive ELISA has the ad-

vantage that the conjugates included should react with all mammalian immunoglobulin G antibodies,[1,4,5] although there have been no specific studies for the species mentioned.

Of 137 samples, only one red deer sample from Müritz National Park was positive, with a mean value of 36.15% (95% confidence interval, 0.1–4; P=0.0072). The competition percentage (S/N%) was calculated: $S/N\% = OD_{sample}/OD_{negativecontrol}$ X 100; where OD_{sample} is the mean optical density value recorded at 450 nm and $OD_{negativecontrol}$ is the mean value of the negative control, read in a microplate reader. Samples less than or equal to 45% were considered positive. We calculated a false-negative and false-positive rate of 1.01% and 4.65% respectively for the assay.[4] The assay has been successfully used in influenza epidemiological studies in multiple species.[1,5] If some samples tested represent false negatives, antibody titers would have to be extremely low to result in false negatives, which would still support infrequent and very low exposure rates of German cervids by IAV. Similarly, we cannot absolutely rule out that the positive sample is not a false positive given the prevalence is below the false-positive rate. However, neither the false-negative nor false-positive rate in the context of these results would suggest anything other than that influenza exposure is negligible in the deer tested.

IAV causes disease in birds and mammals and several strains are zoonotic. Worldwide surveillance focuses on wild and domestic avifauna, pigs, and equids because of zoonotic potential and the economic losses associated with domestic animal infections. Nonetheless, non-avian influenza epidemiology remains largely unexplored. There is recent evidence that New World bats are infected with H17 and H18 strains that are not found in wild or domestic birds, supporting mammalian strain-specific reservoir status.11 Equine influenza virus has jumped to dogs, suggesting that avianindependent transmission of influenza strains may be underestimated.[7]

Older studies [2,12] demonstrated that wild cervids are exposed to influenza strains H1N1 and H3N2 (A/Johannesburg/82/96, A/Sydney/05/97, A/ Victoria/13/75, A/Texas 1/77, and A/USSR 90/ 77), important seasonal human flu strains that have caused pandemics, as well as influenza B virus (B/Beijing/184/93-like). Captive cervids can also be infected with H1N1, H3N2 (A/ brazil/11/78, A/Philipp/2/82), and H4N6 (A/ duck/Czech/56) viruses.[10] Wild cervids, particularly in temperate regions, are sympatric with migrating waterfowl, which could expose them to IAV much like other sympatric mamma-

lian species. However, cervids have not been examined for their prevalence and role in influenza A dynamics. The results of the current study suggest that any role wild cervids play in influenza A dynamics is minor and that the deer species tested are generally not exposed frequently to the virus or are able to resist infection. With the discovery of new influenza viruses, such as influenza D virus, and their circulation in domestic ungulates,[9] further studies should test the role of wildlife, especially cervids, in the epidemiology of these newly discovered pathogens

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Chapter 5
DISCUSSION

Avian Influenza viruses have been a scientific focus for a very long time due to their zoonotic potential and their evolutionary history. Their potential to travel and spread among continents, change and adapt to new hosts, and their ongoing evolution makes them important to human and animal health. Although there have been some reports of wild mammal infections, existing research on the epidemiology, ecology and viral transmission in wild mammals is limited. This thesis aimed to provide serological evidence for exposure of different wild mammals to IAV and identify factors influencing viral prevalence and diversity.

Summary of findings

In Chapter 2 we investigated the role of 14 African wild mammals in IAV ecology testing if phylogeny, sociality or diet influence the viral prevalence and diversity in these species. Our results revealed that carnivores, which commonly include birds in their diet, have significantly higher exposure and diversity of influenza viruses. These results provide plausible evidence that carnivores that actively hunt and consume wild birds are susceptible to AIV and could represent natural or intermediate hosts of influenza viruses. Surprisingly phylogenetic relationship and sociality showed little effect regarding influenza exposure and prevalence, while diet leads to an increased AIV exposure and higher diversity. Furthermore, it demonstrated the complexity of pathogen-host distribution, adaptation, and evolution in a diverse habitat, such as the Namibian savannah-steppe environment.
In Chapter 3 the importance of disease surveillance in a vastly different landscape, such as the Mongolian Gobi Desert was demonstrated. Wild mammalian species share their pastures with other free-ranging wild mammals, migratory birds, and human pastoralists and their livestock. The focus of this chapter was on Asiatic wild asses and their exposure to EIV, which is known to circulate in the area. Mongolian domestic horses exceed 3.6 million individuals and are characterized by an extremely low vaccination rate, and a known endemic circulation of the virus. A significant finding in the ecology and epidemiology of influenza viruses in wild asses was the serological confirmation of H7N7 EIV, although it was presumed to be extinct and has not been isolated since the 1980's. Furthermore, the exposure evidence of Khulans to different AIV, such as H1, H5, H8, and H10 identified, confirms that more in-depth epidemiological surveillance is warranted to better understand the epidemiology and ecology of IV in equids across the Mongolian steppe and their potential role as an alternative route of infection for other wild and domestic species.
Evidence of exposure to IAV has been scarce in the Cervidae Family. Although wild ruminants are not considered to be natural hosts of human in-

fluenza infections, previous studies have shown an intriguing potential when influenza A and B human strain infections was observed in these species. A more comprehensive One Health approach and surveillance would help to clarify the role of these species in influenza epidemiology. As demonstrated in the previous three chapters, influenza viruses are present among different hosts and geographical locations. The prevalence of the virus correlates with the presence or absence of migratory aquatic wild birds, as well as with the diversity and contact with other domestic or wildlife species. However, from the serological surveillance results we obtained from the German National Parks, all three deer species seem to play a minor role in influenza ecology.

Reconsidering influenza A virus ecology

Influenza A virus is one of the most significant emerging infectious diseases. The ecology of these viruses on a global scale is still not fully understood. Although it is widely accepted that aquatic wild birds are the primary natural hosts of influenza A viruses, these constantly evolve which promotes further adaptation to numerous wild and domestic hosts, both avian and mammalian. Land use change, agricultural intensification and expansion, habitat destruction, and fragmentation paired with climate change are factors driving influenza ecology and epidemiology. Globalization of trade promotes disease spread to new naïve populations (Lindahl et al. 2015). Furthermore, influenza viruses may find more fruitful ground for co-existence and therefore genetic reassortment, generating new novel viral variants that can pose a threat to public health. For example, expansion of agricultural practices may bring domestic and wild animals closer, increasing the risk spillover (Jones et al. 2012). Wet markets are suspected to play an important role in viral evolution of AIV, specifically HPAIV. Poultry and waterfowl (ducks and geese) are stocked in markets at high densities, offer optimal conditions for viral exchange and evolution of novel variants (Fournie et al 2012). There are similar consequences as a result of urbanization and human population growth. Increased size of populations may increase disease transmission and risk due to the selective pressure of pathogens (Daszak et al. 2001). Increased human populations consequentially mean increased food production, achieved by modern agricultural practices. High densities of animals may favor disease transmission and adaptation. Low quality animal husbandry and poor health services, decreases the immune system, and increases animal stress, making them more susceptible to disease (Peterson et al. 1991). Furthermore, distribution of the goods requires movement, which further increases the chances of disease transmission to naïve or new populations (Jones et al. 2012). Additionally, low biosecurity measures, especially in small backyard farms in developing countries, increase the exposure exponentially and therefore multiply the risk

of disease (Conan et al. 2012).
Another stressor is habitat destruction, which affects not only migratory birds and wild mammals but humans as well. Loss of habitat has a variety of effects. Migratory birds may lose their stopover, wintering, and breeding grounds, a threat not only to their conservation status but also a challenge concerning disease transmission (Kirby et al. 2008). Various outbreaks have been reported in such sites, with significant impacts to some bird populations (Boere et al. 2006). The decrease of bird habitat has an immediate effect on other wild and domestic birds and mammals, for example due to dilution of biodiversity and increase risk of disease in the remain bird- and animal populations (Luis et al. 2018), and natural pest control (Andy and Elmberg 2014). Animal congregation leads higher contact rates between different species, giving IAV the opportunity to evolve and adapt to new hosts (McCallum and Dobson 2002, Vandegrift et al. 2010). Furthermore, climate change may contribute to disease transmission, by shifting the natural hosts and/or vectors geographical distribution (Rocque et al. 2008), (Epps et al. 2004), or lead to thermal stress as is currently observed in amphibians (Harvel et al. 2002). Although today the most substantial evidence of climate change impacts on disease patterns comes from vector-borne diseases (Harvell et al. 2009), infectious diseases that can persist in the environment and be transmitted environmentally, such as influenza virus (Brown et al. 2009), may show an enhanced transmission potential (Lafferty 2009).

Re-considering influenza A surveillance

The World Organization for Animal Health (OIE) and the World Health Organization (WHO), focus on AI types H5 and H7, in their surveillance. Both institutions gather and analyze virological data on influenza viruses around the world, reporting strategies and measures necessary for avoiding a new pandemic. The surveillance and preventive measures include birds (domestic and wild), poultry, and equids. From a notifiable disease and economic point of view that might be justified, but this surveillance is missing a key component in respect to public health: wildlife. It has been estimated that the majority of zoonotic diseases, including influenza viruses, have a wildlife origin (Taylor et al. 2001). While the importance of wildlife disease is slowly gaining more attention, a more strategically and One-Health based surveillance approach is needed. A One-Health tactic is particularly relevant where wild and domestic animal, human, and environmental health are connected (Martin et al. 2011, Wiethoelter et al. 2015, Hassell et al. 2017). Passive as well as active surveillance has to be implemented. Targeted hosts have to be carefully selected. These might not always be the final species of interest, but rather a sympatric species or one in close contact that can be readily

sampled. The success of this approach has been demonstrated for domestic dogs to assess to what extent sympatric wild carnivores are at risk from Canine distemper virus exposure (Cleaveland et al. 2000). Similarly, we show that carnivores feeding on, or scavenging birds, have a higher exposure to influenza viruses. In summary, interdisciplinary collaboration of wildlife veterinarians, biologists, epidemiologists, and other health science professionals, policymakers and local communities, environmentalists and sociologists, will be necessary for a successful outcome.

Concluding remarks

Influenza viruses continue to show a remarkable genetic evolution and expansion of host species. Continued and combined surveillance of both domestic and wild animals is essential to better understand their pandemic potential and the possibility of human infection. Moreover, research concerning influenza virus ecology and the interactions dynamics with new hosts would make a significant contribution to the development of further prevention and control measures. Multi- and interdisciplinary collaboration is needed to achieve such a goal. This must be paired with different serological and molecular methods to elucidate the evolution and origin of the pathogens subtypes. In conclusion, a more holistic and One Health approach with emphasis on an interdisciplinary collaboration could help clarify the complex nature of Influenza A viruses.

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Summary

Emerging infectious diseases, particularly zoonotic diseases, have been the focus of scientific and public interest in recent years. Influenza A viruses (IAV), currently and historically have been zoonotic agents of great importance for both human and animal health. There is evidence of infection and exposure of numerous avian and mammalian species.
Furthermore, studies have shown the potential of influenza viruses to cross species barriers, infecting many domestic and wild mammalian species. This thesis investigated exposure of multiple wild mammalian species to IAV to assess their potential role in the transmission and evolution of the viruses using serological methods. A number of factors were identified that promote influenza transmission and exposure. Contact between domestic and wildlife species, such as Asiatic wild asses with their sympatric relatives, was one factor. Furthermore, animals with both avian and mammalian influenza receptors in their respiratory tract, such as Equids, are more susceptible to influenza A virus infection, as we demonstrate in Chapter 3. The same effect is demonstrated in Chapter 2, where we showed that carnivores that consume birds had a higher diversity and greater exposure to AIV, while sociality and phylogenetic relationship does not seem to drive influenza exposure.
If their susceptibility remains a dead-end infection, or are we facing a new endemic, and therefore potentially epidemic or pandemic infection, remains an open question. Serological surveys provide information about past infections, but molecular methods are needed in order to draw conclusions on the evolution and adaptation of the viruses in these potential new hosts. Integration of lo pathogenic IAV, LPAIV, in epidemiological studies, as well as IAV that are thought to no longer circulate, like H7N7 in equids, is of great importance. Wildlife may represent an unrecognized ecological niche for IAV. Additionally, constant and rapid change in the environment, such as climate change, agriculture practices, and habitat destruction, are influencing hosts, pathogens, and diseases and must be taken in account in wildlife disease surveillance studies. Integration and analysis of data from all these different sources, including animal and human data, will give us the tools to perform risk analysis, and apply possible control or prevention schemes.

Zusammenfassung

Seroprävalenz von Influenza-A-Viren bei wilden Säugetieren

Aufkommende Infektionskrankheiten, insbesondere Zoonosen, standen in den letzten Jahren im Mittelpunkt des wissenschaftlichen und öffentlichen

Interesses. Influenza-A-Viren (IAV) sind derzeit und in der Vergangenheit Zoonosenerreger von sehr großer Bedeutung für die Gesundheit von Mensch und Tier. Es gibt Hinweise auf eine Infektion und Exposition zahlreicher Vogel- und Säugetierarten.
Darüber hinaus haben Studien das Potenzial von Influenzaviren aufgezeigt, Artenbarrieren zu überwinden und viele Haus- und Wildsäugetierarten zu infizieren. Diese Arbeit untersuchte die Exposition mehrerer wilder Säugetierarten gegenüber der IAV, um ihre potenzielle Rolle bei der Übertragung und Evolution der Viren mit serologischen Methoden zu bewerten. Es wurden eine Reihe von Faktoren identifiziert, die die Übertragung und Exposition der Grippe fördern. Der Kontakt zwischen Haus- und Wildtierarten, wie z.B. dem asiatischen Wildesel mit seinen sympatrischen Verwandten, war ein Faktor. Darüber hinaus sind Tiere mit Influenza-Rezeptoren sowohl für Vögel als auch für Säugetiere in ihren Atemwegen, wie beispielsweise Equiden, anfälliger für Influenza-A-Viren, wie wir in Kapitel 3 zeigen. Der gleiche Effekt wird in Kapitel 2 gezeigt, wo wir dargestellt haben, dass Fleischfresser, die Vögel konsumieren eine höhere Vielfalt und eine größere Exposition gegenüber AIV hatten, während Sozialität und phylogenetische Beziehung die Influenza-Exposition nicht zu beeinflussen scheinen.
Ob ihre Anfälligkeit eine Sackgasseninfektion bleibt oder ob wir vor einer neuen endemischen und damit potenziell epidemischen oder pandemischen Infektion stehen, bleibt eine offene Frage. Serologische Untersuchungen liefern Informationen über frühere Infektionen, aber molekulare Methoden sind notwendig, um Rückschlüsse auf die Evolution und Anpassung der Viren in diesen potenziellen neuen Wirten zu ziehen.
Die Integration von niedrig pathogenen IAV, LPAIV, in epidemiologischen Studien sowie von IAV, von denen angenommen wird, dass sie nicht mehr zirkulieren, wie H7N7 bei Equiden, ist von großer Bedeutung. Wildtiere können für die IAV eine nicht anerkannte ökologische Nische darstellen. Darüber hinaus beeinflussen ständige und schnelle Veränderungen in der Umwelt, wie Klimawandel, landwirtschaftliche Praktiken und die Zerstörung von Lebensräumen, Wirte, Krankheitserreger und Krankheiten und müssen bei Studien zur Überwachung von Tierkrankheiten berücksichtigt werden. Die Integration und Analyse von Daten aus all diesen verschiedenen Quellen, einschließlich tierischer und menschlicher Daten, wird uns die Werkzeuge an die Hand geben, um Risikoanalysen durchzuführen und mögliche Kontroll- oder Präventionsverfahren anzuwenden.

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Acknowledgments

Looking back into these 4 years, I can only feel grateful for the journey, the lesson learned, and the people that were on my side all along. My written acknowledgment can only express a small percentage of what I really feel. You all made me a better person and a better scientist, and for that I can only be grateful.

Starting with the two people who believed I could do it, and with their help I did: My supervisors and mentors Alex Greenwood and Chris Walzer. I met both during my undergraduate studies, and only in my wildest dreams I would be part of a team including both of them. Well, as a helpless romantic, I can only advice that we should all keep our dreams alive. Thank you both for every amazing discussion, for sharing your knowledge, for your guidance and support, for the laughter and joy, for the understanding and mentoring all of these years! You gave me freedom for personal and professional growth, but you were always by my side. I couldn't be luckier than that! I am sure that our paths will cross again and I am looking forward to it!

A special thanks is for Gábor Czirják! You were never official my supervisor, but your support was equally big and important! I couldn't thank you enough for your help, your understanding, your thoughts, and support, for having always an open door for me and the patients to hear me out! I loved our collaborations and I am looking forward to the next ones! Thank you, thank you and THANK you!

Moreover, I want to thank all the members of the Wildlife Disease Department. Karin and Katja, your support is amazing! Claudia… Without you life in IZW would be bitter! I am so grateful for your existence! I want to thank all my fellow PhD students and Postdocs! One of the most important and amazing

things in life is compassion, support, and understanding! Not only I had all of that, but also I gained friendships for life! Sonia, Nicollo, Tanja, Deniz, Renata, Peter, and Ulrike you all have a special place in my heart! I wish the future would bring me equally awesome colleagues and friends like you!
My special thanks to all the co-authors of my papers! Thank you for your contribution, comments, and expertise and for making me a better scientist. I couldn't have done it without you! Particular I want to thank my fellow expedition companions. Petra, John, Nikolaus, Chris, Diana, Thierry and all the rest, thank you for the awesome times out in the field! I will never forget the laughter's during the successful, but also the difficult times, the adventures, and the talks that we shared! Who would say that a ducked-taped helicopter ride could be so much fun?

Special thanks are going to the people behind the 'scientific' scene. My family! The biggest gift and bliss in my life! This is my unique gift, my power engine, my everything!! Mom, Raschmie, Venthesikimi and Alexandros, what would life be without you? You give meaning to my work and joy to my success. I thank God every day for having you! Jörn, your continuous support, patience, and love guided me through some difficult times… Thank you for being here!

“To develop a complete mind: Study the science of art; Study the art of science. Learn how to see. Realize that everything connects to everything else.”
— Leonardo da Vinci

Finanzierungsquellen – Funding Sources
Die Arbeiten wurden finanziell unterstützt durch Leibniz Gemeinschaft (SAW-2015-IZW-1 440).

Interessenskonflikte – Conflict of Interest
Es besteht kein Interessenskonflikt durch finanzielle Unterstützung der Arbeiten.

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Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.
I hereby confirm that I did all this work myself.