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MAJOR ARTICLE

Diet May Drive Influenza A Virus Exposure in African Mammals

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(See the Editorial commentary by Root, on pages 169–71.)

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 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

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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.

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

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] in R statistical software [33].

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

Information 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%.

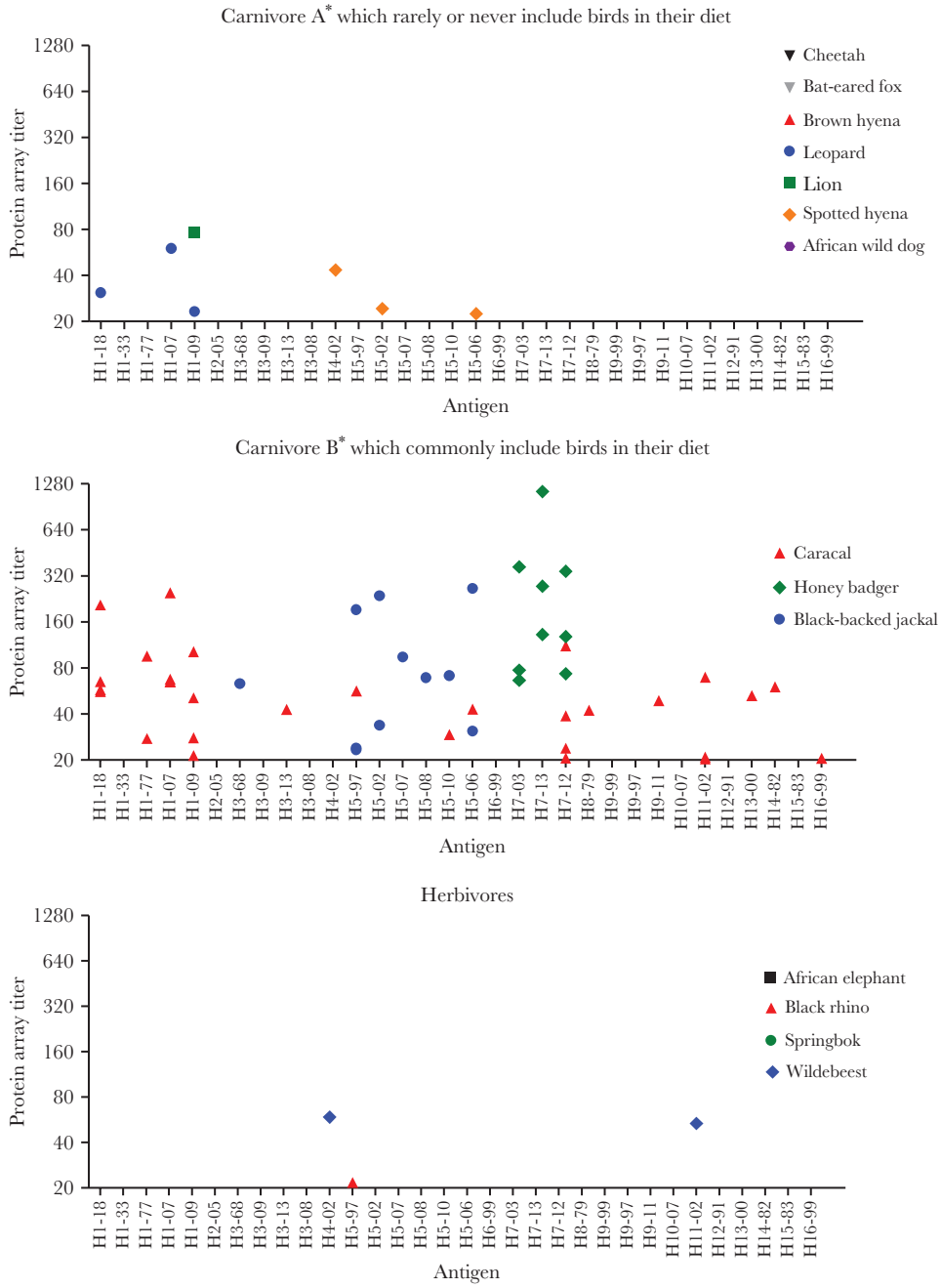

Figure 1. Overview of protein microarray titers for all hemagglutinin (HA) part antigens, by diet and species. Antigens are abbreviated as described in Supplementary Table 1. The lower limit of detection was a titer of 20; titers <20 were regarded as negative findings and are not depicted. *The carnivore 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).

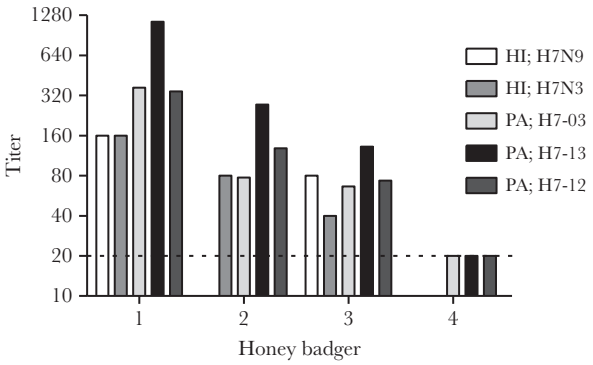

Figure 2. Hemagglutination inhibition (HI) assay titers, using 2 different influenza virus strains, and protein microarray (PA) titers, using 3 different influenza virus strains, for 4 honey badgers. Dotted line is the starting dilution for the protein microarray.

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

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 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 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).

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.

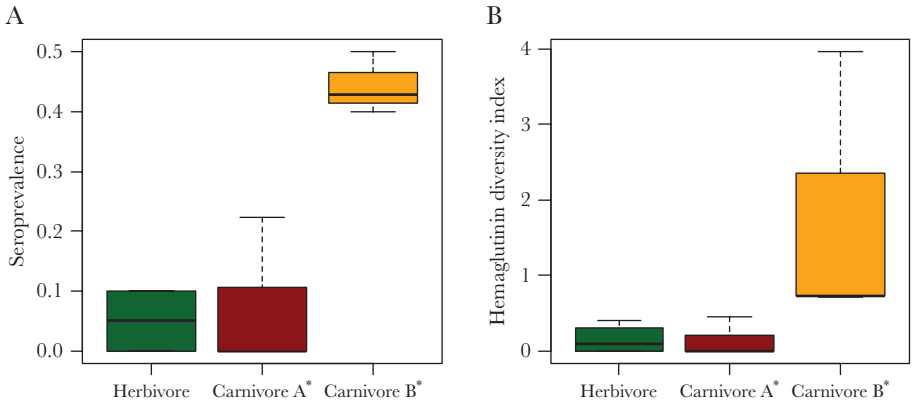

Figure 3. Box plots illustrating the relationships between diet and influenza A virus seroprevalence (*A*) and hemagglutinin diversity (*B*). To correct for variation in sample sizes among species, the hemagglutinin diversity index for each species represents the number of hemagglutinin subtypes that 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).

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

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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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