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Serologic Evidence of West Nile Virus Infections in Wild Birds Captured in Germany

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Abstract. To assess the risk of acquiring a West Nile virus (WNV) infection in Germany, we investigated samples from migrating and from resident birds. Because of their stay in or migration through WNV-endemic regions, these birds are at risk to become infected with WNV. Blood samples from 3,399 birds, representing 87 bird species, were collected in Germany in 2000 and in 2002–2005. Overall, 53 birds belonging to 5 species had WNV-neutralizing antibodies. Fifty-nine birds belonging to 9 species were reactive by WNV immunofluorescence assay, and 8 birds had neutralizing antibodies against Usutu virus. Because of maternal antibody transfer via egg yolk, WNV-antibody titers in white stork nestlings were generally lower than those in adults. Despite a relatively high percentage of stork nestlings with antibodies, no viral genomes were detectable by polymerase chain reaction. In Germany, the prevalence of antibodies to WNV in migrating birds wintering in Africa or southern Europe is comparatively low.

INTRODUCTION

Within the genus *Flavivirus* of the family *Flaviviridae*, West Nile virus (WNV) and Usutu virus (USUV) are grouped to the Japanese encephalitis antigen group. West Nile virus is endemic in different parts of the world, whereas USUV seems to be endemic only in Africa and possibly in Austria.[1,2] After its introduction into New York City in 1999, WNV gained worldwide interest. In the immunologically naive host population, it has spread rapidly across North America and has had an enormous impact not only on avian and equine species but also on humans, especially in the United States. West Nile virus is transmitted naturally by ornithophilic mosquitoes (particularly species of the genus *Culex*) within the bird population, but some mosquitoes are also capable of transmitting the virus to mammals such as horses or humans.[3,4] The virus has been found in more than 150 species of wild and domestic birds in the United States.[5]

Millions of birds migrate every year between Europe and Africa, wintering in or passing through WNV-endemic areas. Bird migration seems to play a major role in the dissemination of WNV and USUV.[6,7] In Europe, antibodies to WNV have been detected in several bird species.[8,9] In the United Kingdom, investigations on the prevalence and incidence of WNV infections were carried out in 2001–2002 and showed that migrating and resident birds had antibodies to WNV or viral genomes.[10] In France, virus isolation was successful in various samples from mammals such as horses.[3] There are no data on endogenous WNV and USUV infections in Germany, whereas USUV infections have been diagnosed in neighboring Austria.[2] The aim of the present study was to investigate migrating and resident birds as a potential reservoir of WNV and USUV by using serologic and molecular methods, and to assess the possibility that migrating species import these viruses from their wintering areas in Africa.

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MATERIALS AND METHODS

Bird capture and sampling. In 2000 and in 2002–2005 3,399 samples, representing 87 species of migrating and resident birds (Table 1), were collected in the context of bird-ringing activities or other research interests. The birds were captured by traps and mist nets, bled, and released or downed in pest-control programs. Sampling of white stork nestlings and raptor nestlings was performed by bleeding nestlings at nest sites. Morphologic examinations were performed to determine sex, age, and species. Rings engraved with a serial number from German ringing centers were used for individual marking. Several samples were collected from injured wild birds during veterinary care. National laws and regulations regarding protection, conservation, and animal welfare were respected.

Study sites. The study sites were spread all over Germany and located in rural or agricultural areas (Figure 1).

Sampling. Blood was obtained by puncturing the *vena ulnaris*. The volume of blood was variable depending on the bird species (between 10 μL and 1 mL). Blood was collected in microtubes for serum separation (Sarstedt, Nuembrecht, Germany), in monovettes containing EDTA (Sarstedt), or in reaction tubes with anticoagulant preservative buffer (10 mM Tris-HCl, pH 7.5, 1.0 M NaCl, 0.1% Tween 20). Body fluids from dead birds were collected from the carcasses. Sera and plasma were stored at -70°C until use.

Diagnostic assays. Two methods were used for serologic diagnosis: an indirect immunofluorescence assay (IFA) and a neutralization test (NT). The first screening was done using a WNV IFA kit (EUROIMMUN, Lübeck, Germany) following the manufacturer's instructions. The samples were diluted 1:10 with sampling buffer and 25 μL of the diluted samples were applied to a biochip slide and incubated for 30 minutes at room temperature. After the slide was washed for 10 minutes, a non-labeled rabbit anti-dove IgG hyperimmune serum diluted 1:300 was added to the biochips and incubated for 30 minutes. The rabbit anti-dove IgG reacted with a broad spectrum of antibodies derived from different bird species (Grund C, unpublished data). After a second washing step, 25 μL of

TABLE 1
Eighty-seven species of resident, migrating, and wintering birds investigated*

Order	Common name	Species	Samples investigated by NT†	Samples investigated by IFA	Migration status
Anseriformes	Mute swan	*Cygnus olor*	125	125	RMW
	Bean goose	*Anser fabalis*	7	7	MW
	White-fronted goose	*Anser albifrons*	109	109	MW
	Pink-footed goose	*Anser brachyrhynchus*	1	1	MW
Ciconiiformes	White stork	*Ciconia ciconia*	569	569	M
Accipitriformes	Osprey	*Pandion haliaetus*	140	140	M
	Honey buzzard	*Pernis apivorus*	2	2	M
	Marsh harrier	*Circus aeruginosus*	0	23	M
	Goshawk	*Accipiter gentilis*	19	19	R
	European sparrowhawk	*Accipiter nisus*	1	2	RMW
	Black kite	*Milvus migrans*	1	1	M
	White-tailed sea eagle	*Haliaeetus albicilla*	36	36	RW
	Common buzzard	*Buteo buteo*	41	41	RMW
Falconiformes	Peregrine falcon	*Falco peregrinus*	3	3	RMW
	Common kestrel	*Falco tinnunculus*	10	10	RMW
Cuculiformes	Cuckoo	*Cuculus canorus*	0	2	M
Strigiformes	Barn owl	*Tyto alba*	2	2	R
	Little owl	*Athene noctua*	0	12	R
	Scops owl	*Otus scops*	0	1	A
	Long-eared owl	*Asio otus*	6	6	RMW
	Tawny owl	*Strix aluco*	6	6	R
Coraciiformes	Kingfisher	*Alcedo atthis*	0	1	RMW
Piciformes	Green woodpecker	*Picus viridis*	0	1	R
	Great spotted woodpecker	*Dendrocopos major*	0	4	RW
Passeriformes	European magpie	*Pica pica*	0	5	R
	Jay	*Garrulus glandarius*	0	10	RMW
	Red-backed shrike	*Lanius collurio*	5	26	M
	Rook	*Corvus frugilegus*	14	14	RMW
	Carrion crow	*Corvus corone*	9	9	R
	Raven	*Corvus corax*	3	3	R
	Blue tit	*Parus caeruleus*	0	51	RMW
	Great tit	*Parus major*	3	121	RMW
	Crested tit	*Parus cristatus*	0	8	R
	Coal tit	*Parus ater*	0	16	RMW
	Marsh tit	*Parus palustris*	0	3	R
	Willow tit	*Parus montanus*	0	4	R
	Sand martin	*Riparia riparia*	0	29	M
	Swallow	*Hirundo rustica*	0	3	M
	House martin	*Delichon urbicum*	0	19	M
	Long-tailed tit	*Aegithalos caudatus*	0	2	RMW
	Wood warbler	*Phylloscopus sibilatrix*	4	7	M
	Willow warbler	*Phylloscopus trochilus*	44	135	M
	Chiffchaff	*Phylloscopus collybita*	13	52	M
	Grasshopper warbler	*Locustella naevia*	0	3	M
	Sedge warbler	*Acrocephalus schoenobaenus*	0	9	M
	Marsh warbler	*Acrocephalus palustris*	0	70	M
	Reed warbler	*Acrocephalus scirpaceus*	0	306	M
	Great reed warbler	*Acrocephalus arundinaceus*	0	8	M
	Icterine warbler	*Hippolais icterina*	1	47	M
	Melodius warbler	*Hippolais polyglotta*	0	1	M
	Blackcap	*Sylvia atricapilla*	0	43	M
	Garden warbler	*Sylvia borin*	2	149	M
	Lesser whitethroat	*Sylvia curruca*	14	32	M
	Common whitethroat	*Sylvia communis*	4	30	M
	Goldcrest	*Regulus regulus*	0	1	RMW
	Firecrest	*Regulus ignicapillus*	0	2	M
	Nuthatch	*Sitta europaea*	0	3	RMW
	Short-toed treecreeper	*Certhia brachydactyla*	2	5	R
	Wren	*Troglodytes troglodytes*	1	17	RMW
	Starling	*Sturnus vulgaris*	0	20	RMW
	Blackbird	*Turdus merula*	9	184	RMW
	Fieldfare	*Turdus pilaris*	0	2	RMW
	Song thrush	*Turdus philomelos*	2	34	M
	Redwing	*Turdus iliacus*	0	1	MW
	Pied flycatcher	*Ficedula hypoleuca*	7	24	M
	Spotted flycatcher	*Ficedula striata*	0	26	M
	Whinchat	*Saxicola rubetra*	0	20	M

(continued)

TABLE 1
Continued*

Order	Common name	Species	Samples investigated by NT †	Samples investigated by IFA	Migration status
Passeriformes	Robin	*Erithacus rubecula*	22	160	RMW
	Thrush nightingale	*Luscinia luscinia*	1	1	M
	Nightingale	*Luscinia megarhynchos*	0	18	M
	Bluethroat	*Luscinia svecica*	0	13	M
	Black redstart	*Phoenicurus ochruros*	0	31	M
	Redstart	*Phoenicurus phoenicurus*	9	52	M
	Dunnock	*Prunella modularis*	4	36	RMW
	House sparrow	*Passer domesticus*	0	41	R
	Tree sparrow	*Passer montanus*	0	43	R
	Tree pipit	*Anthus trivialis*	14	31	M
	Yellow wagtail	*Motacilla flava*	1	41	M
	White wagtail	*Motacilla alba*	1	2	MW
	Chaffinch	*Fringilla coelebs*	2	35	RMW
	Bullfinch	*Pyrrhula pyrrhula*	0	24	RMW
	Common crossbill	*Loxia curvirostra*	0	13	RMW
	Greenfinch	*Carduelis chloris*	0	154	RMW
	Siskin	*Carduelis spinus*	0	1	RMW
	Yellowhammer	*Emberiza citrinella*	0	2	RMW
	Ortolan bunting	*Emberiza hortulana*	0	1	M
	Reed bunting	*Emberiza schoeniclus*	0	5	MW

* NT = neutralization test; IFA = indirect immunofluorescence assay; R = resident; M = migratory; W = winter guest; A = anomaly in Germany, since 1980 less than 5 individuals were verified.[11,12]

† Number tested in IFA is not always identical with the number of samples tested in NT because of too low amounts of sample volume or high toxicity of the sample in cell culture.

a mixture of a goat anti-bird fluorescein isothiocyanate (FITC)–labeled antibody (Bethyl Inc., Montgomery, AL) diluted 1:50 in phosphate-buffered saline (PBS) plus Tween 20 and an anti-rabbit FITC-labeled antibody (Dianova, Hamburg, Germany) for detection of the rabbit anti-dove IgG hyper immune serum diluted 1:300 were applied to the biochips. After a second incubation for 30 minutes and a washing step, the slide was embedded with a drop of glycerol/PBS according to the manufacturer's instructions and analyzed under a fluorescence microscope (Axioskop MC80, filters 495 nm and 528 nm; Zeiss, Oberkochen, Germany). Reactive samples were titrated in dilution steps of 1:10, 1:100, and 1:1000 according to the manufacturer's instructions. Reference goose serum with a known titer of antibodies to WNV was used as a control sample.

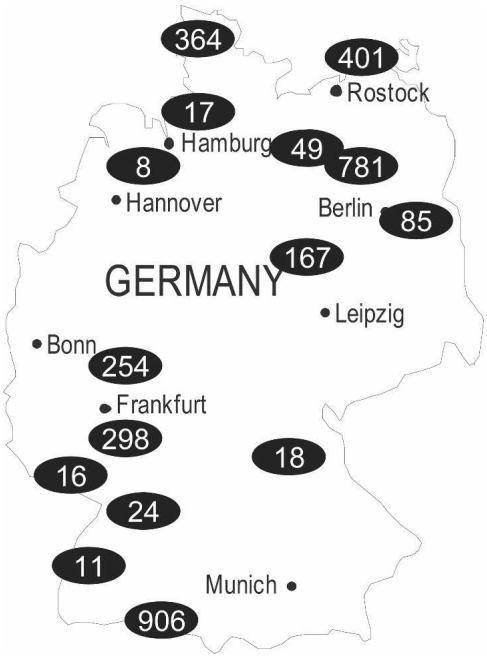

FIGURE 1. Map of Germany showing different sampling sites. Numbers in ellipses indicate the number of captured birds.

The method of choice for flavivirus diagnostics and differentiation is the virus NT. A WNV-specific NT was performed in a 96-well plate format (Nunc, Wiesbaden, Germany). Vero cells were grown in Eagle's minimal essential medium (MEM) (PAA Laboratories, Coelbe, Germany) supplemented with 10% fetal bovine serum (PAA Laboratories) in 96-well microtiter plates. Samples were diluted 1:10 in PBS. The stock solution of WNV strain Israel was diluted in Eagle's MEM with 1% ciprofloxacin (PAA Laboratories) to a constant concentration of 500 50% tissue culture infective doses ($TCID_{50}$)/mL. A total of 25 μL of virus suspension were mixed with 25 μL of serum or plasma dilution and incubated for 1 hour at 37°C. After incubation, the inoculum of serum and virus was added to the cells and incubated for 3 days at 37°C in an atmosphere of 5% CO_2. The suspension of plasma and virus was then removed from the cells and 100 μL of fresh medium were added to each well and incubated for 3 days at 37°C in an atmosphere of 5% CO_2. After incubation, the cells were fixed and stained with naphthalene black and analyzed under a light microscope. Neutralizing goose sera were included in all experiments. Each test sample was investigated in duplicate with a single well of serum-cell control without virus. Samples with neutralizing antibodies at a dilution of 1:10 were titrated (two-fold serial dilutions from 1:10 to 1:2,560) to determine end point titers for WNV. Antibody titers were determined as the highest dilution of serum or plasma at which 50% of the wells did not show a cytopathic effect. Samples with a WNV antibody titer ≥ 10 were considered positive because serum dilutions $< 1:10$ were often toxic for cells.

Some samples were further investigated in a USUV-specific plaque reduction neutralization test (PRNT). The test

TABLE 2
Oligonucleotide primers and TaqMan probe for reverse transcription–polymerase chain reaction of WNV and SMRV*

	Sequence (5′ → 3′) of primer and TaqMan probe	Orientation‡	Genome position WNV: AY532665, SMRV: M23385
ProC-F1	CCTgTgTgAgCTgACAAACTTAgT	S	10–33
ProC-R	gCgTTTTAgCATATTgACAgCC	AS	132–153
ProC-TM†	6FAM-CCTggTTTCTTAgACATCgAgATCTXCgTgCp	AS	89–113
SMRV-7489 F	CCT gCT AgT Agg ATT ggg TgT CTC T	S	7489–7513
SMRV-7658 R	CTA CTT Cgg CTA ggg AAT CTA gTT g	AS	7634–7658
SMRV-env-probe	6FAM-TAA CgA CgT CCA AgC CTT gTC Tag CAC CAXT-p	S	7588–7617

* WNV = West Nile virus; SMRV = squirrel monkey retrovirus.
† X = carboxytetramethylrhodamine; FAM = 6′-carboxyfluorescein; p = phosphate.
‡ S = sense; AS = antisense.

procedure was performed according to published studies[13,14] with slight modifications. The test was performed in 48-well microtiter plates (Nunc) with Vero cells cultivated in Eagle's MEM (PAA Laboratories) with 10% fetal bovine serum (PAA Laboratories) and 1% ciprofloxacin (PAA Laboratories). The serum or plasma samples were diluted two-fold from 1:10 to 1:640 in PBS. Aliquots (50 μL) of USUV strain Vienna 2001 from Austria containing 150 $TCID_{50}$/mL were added to 50 μL of the sample and incubated for 1 hour at 37°C. The virus-sample suspension was applied to the cells and incubated for 1 hour at 37°C. After incubation, the mixture of plasma and virus was removed from the cell layer and fresh medium was added. An overlay of carboxymethyl cellulose medium (BDH Ltd., Poole, United Kingdom) was added to the cells and the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 3 days. The plates were fixed and stained with naphthalene black. Plaques were counted and the 50% PRNT ($PRNT_{50}$) titer was calculated according to Reed and Münch.[15]

Viral RNA was isolated from 552 samples from white storks; 100 μL of plasma was centrifuged for 1.5 hours at 14,000 rpm. The pellet was used for virus extraction and the supernatant was used for serologic testing. RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was eluted in 60 μL of RNase-free water (Fluka Chemikalien GmbH, Buchs, Switzerland) supplemented with RNA (100 ng/μL) (Roche, Mannheim, Germany). RNA was stored at –70°C before further use.

cDNA was synthesized by reverse transcription of 11.6 μL of extracted RNA in a 20-μL reaction volume. For transcription, 1 μL of the specific reverse primer (10 μM; Table 2) and 1 μL of reverse transcriptase (200 U/μL) (Invitrogen, Karlsruhe, Germany) were used. The cDNA was stored at –20°C before further use.

Reverse transcription–polymerase chain reaction (RT-PCR) was performed on a gene sequence of the capsid protein and 5′-untranslated region using the TaqMan method (Table 2). As an internal control standard of the RNA extraction, of cDNA synthesis, and PCR, plasma was spiked with a constant volume of heat-inactivated supernatant of Squirrel monkey retrovirus (SMRV), family *Retroviridae*. A specific RT-PCR using the SMRV envelope protein sequence was established (Table 2).

RESULTS

Serum or plasma samples (n = 3,399) were collected in 2000–2005. They were derived from 87 different species; specifically in 2000, 41 samples from passerine birds were collected; in 2002, 1,175 samples from passerine birds and waterfowl; in 2003, 438 blood samples from White Storks; in 2004, 1,504 samples from white stork nestlings, passerine birds, and raptors; and in 2005, 241 samples from white storks and raptors, including 106 samples from nestlings and 32 from adult ospreys.

Antibodies against WNV were detected using IFA in nine species (Table 3). Washed samples were analyzed by a WNV NT, neutralizing antibodies to WNV were detected in five species (Table 3). Fifty-nine blood samples from birds had antibodies to WNV by IFA, 27 of which were also positive in the NT. Using the NT, 24 additional sera were identified that were not reactive by IFA.

Because of the small amount of blood from passerine birds, the WNV NT could only be carried out on 191 samples. However, because of the high cytotoxic effect of these samples, only 45 samples gave a reliable result. Of all passeriformes investigated, nine samples were positive by IFA and seven of these samples had neutralizing antibodies (Table 3). On the basis of the IFA results, the percentage of samples with antibodies to WNV was low. Thirty-three (5.8%) of 569 *Ciconia ciconia* samples were reactive by WNV IFA, and only 13 (2.3%) samples were positive by WNV NT. Three sera from *Cygnus olor* showed neutralizing activity, but these sera

TABLE 3
Number of avian species tested positive for antibodies to West Nile virus (WNV) by IFA and NT*

Taxon/species	No. tested in IFA	No. WNV IFA reactive	No. tested in NT†	No. WNV NT positive
Passeriformes				
Erithacus rubecula	160	1	1	–
Ficedula hypoleuca	24	2	2	2
Phoenicurus phoenicurus	52	2	2	1
Lanius collurio	26	2	3	3
Motacilla flava	41	1	1	–
Sylvia borin	149	1	1	1
Ciconiiformes				
Ciconia ciconia	569	33	556	13
Falconiformes				
Pandion haliaetus	140	16	140	28
Milvus migrans	1	–	1	1
Haliaeetus albicilla	36	1	36	–
Accipiter gentilis	19	–	19	1
Anseriformes				
Cygnus olor	135	–	135	3

* IFA = indirect immunofluorescence assay; NT = neutralization test.
† Number of analyzable samples.

TABLE 4
Antibody titers of bird sera by West Nile virus IFA*

Species	IFA titer								Total
	10	32	50	80	100	320	500	1,000	
Haliaeetus albicilla	–	–	–	–	–	–	1	–	1
Pandion haliaetus									
Adults	–	3	–	–	10	1	–	1	15
Nestlings	–	–	–	–	1	–	–	–	1
Total	–	3	–	–	11	1	–	1	16
Ciconia ciconia									
Adults	–	–	–	–	–	1	–	1	2
Nestlings	12	17	–	–	2	–	–	–	31
Total	12	17	–	–	2	1	–	1	33
Sylvia borin	–	–	–	–	1	–	–	–	1
Motacilla flava	1	–	–	–	–	–	–	–	1
Lanius collurio	–	–	1	–	–	–	–	1	2
Ficedula hypoleuca	–	–	2	–	–	–	–	–	2
Erithacus rubecula	–	–	–	–	1	–	–	–	1
Phoenicurus phoenicurus	1	1	–	–	–	–	–	–	2

* Values are number of reactive birds. IFA = indirect immunofluorescence assay.

showed negative results in the WNV IFA. Sixteen (11.4%) of 140 sera of *Pandion haliaetus* were reactive in the WNV IFA. In neutralization assays, the number of positive sera was higher (n = 28, 20%). Only one serum each of samples from *Haliaeetus albicilla* (n = 36), *Milvus migrans* (n = 1), and *Accipiter gentilis* (n = 19) had neutralizing antibodies to WNV.

To gain insight into the potential cross reactivity of bird-infecting flaviviruses, 25 samples reactive against WNV in the IFA but negative in the NT were investigated in the USUV NT. Three samples were positive and had $PRNT_{50}$ titers of 17, 21, and 37, respectively. The antibody titers against WNV varied in different serologic test systems and among the individuals of the species. Tables 4 and 5 show that antibody titers determined using IFA and NT were generally lower in nestlings than in adults (titers of adult white storks were 3-fold to 100-fold higher than in nestlings). In ospreys, only one nestling bird of 106 samples tested was reactive in WNV IFA but not in WNV NT; all other reactive samples were from adult birds.

To clarify whether nestling and adult white storks were infected with WNV, 551 samples were investigated with a WNV-specific RT-PCR. No amplification of viral genomes was observed in the sera or plasma. As an internal control, SMRV was added to each sample as an RNA extraction and PCR control. No inhibition in the process of extraction, cDNA synthesis, and PCR was observed.

Regarding the importation of WNV from Africa to Germany, bird species with WNV-specific antibodies were correlated with their migration status. Five species, most of which were migratory birds, had antibodies against WNV. While *C. ciconia*, *M. migrans*, and *P. haliaetus* migrate to tropical Africa, birds of the species *Cygnus olor* show a more complex migration behavior. *Accipiter gentilis* is a resident bird species, whereas birds of the species *Cygnus olor* are partial migrants.

DISCUSSION

In Germany, only limited information on the prevalence and incidence of WNV infections is available. In a study from Israel, white storks from Germany had WNV-neutralizing antibodies and juveniles on the migration route to Africa passing Israel were diagnosed with WNV.[6] The questions arose where white storks acquire the infection and whether there is evidence that WNV is present in Germany. We investigated the presence of antibodies to WNV in migrating and resident birds in different parts of Germany. A variety of migratory birds that breed in central Europe use African-Eurasian migration routes, which are discussed as routes for importing WNV or other flaviviruses such as USUV into Europe.[6,16] Birds seropositive for WNV were identified across Germany, demonstrating that there is no hotspot of birds with WNV (Figure 1). We identified 5 of 87 species with neutralizing antibodies, 3 of which can be correlated with wetland habitats. In most of the bird species, the percentage with neutralizing antibodies was low, with the exception of ospreys.

We detected specific antibodies in adults and in nestlings. The antibody titer of the nestlings most likely reflects the maternal antibody status because it is well known that maternal antibodies to WNV are transmitted from the mother during egg production.[17,18] We investigated nestlings of ospreys and white storks at 2–9 weeks of age, but only one osprey had

TABLE 5
Antibody titers of bird sera by West Nile virus neutralization test*

Species	NT titer												Total
	10	15	20	30	40	80	160	240	320	640	1,280	2,560	
Pandion haliaetus													
Adults	3	–	2	1	3	6	3	3	6	1	–	–	28
Nestlings	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	3	–	2	1	3	6	3	3	6	1	–	–	28
Ciconia ciconia													
Adults	1	–	–	–	–	–	1	–	–	–	1	–	3
Nestlings	2	7	–	1	–	–	–	–	–	–	–	–	10
Total	3	7	–	1	–	–	1	–	–	–	1	–	13
Cygnus olor	3	–	–	–	–	–	–	–	–	–	–	–	3
Accipiter gentilis	1	–	–	–	–	–	–	–	–	–	–	–	1
Milvus migrans	–	–	–	–	–	1	–	–	–	–	–	–	1
Ficedula hypoleuca	1	1	–	–	–	–	–	–	–	–	–	–	2
Lanius collurio	1	–	1	–	–	–	–	–	–	–	–	–	2
Phoenicurus phoenicurus	1	–	–	–	–	–	–	–	–	–	–	–	1

* Values are number of positive birds.

antibodies to WNV by the IFA. In contrast, we detected neutralizing antibodies in adult birds living in the same region. However, we found a higher percentage of WNV-specific antibodies in nestlings of white storks. These results either reflect differences in the transmission of WNV-specific antibodies via egg yolk or differences in the half-life of maternal antibodies in the offspring. There is limited information on the persistence of maternal antibodies against WNV in birds, which complicates interpretation of serologic results.[18] Viral genomes were not detected in nestling nor adult white storks by PCR, which implies that the antibody response observed in the birds seems to be an indirect marker of the serologic status of the parent birds.

The serologic data obtained by IFA and NT were not congruent in all cases. It is well known that there is high cross-reactivity of closely related flaviviruses in antigen detection systems such as the IFA and an enzyme-linked immunosorbent assay.[19] The method of choice for detecting antibodies against flaviviruses is the NT, but cross-reacting antibodies have also been observed in this assay. High titers of neutralizing antibodies may represent cross-reacting antibodies from flaviviruses, especially of the Japanese encephalitis serocomplex. Differences in antibody titers against different flavivirus isolates can give further information on the virus responsible for the reduction of the immune response. The NT procedure used is very stringent and used a 100% knockout of virus infectivity for antibody detection. Under these highly demanding conditions, it could be assumed that antibodies to WNV were acquired through exposure to WNV.

Three samples of *C. ciconia*, *Phoenicurus phoenicurus*, and *P. haliaetus* showed positive results in the WNV IFA and had low-level neutralizing antibody titers to USUV. Findings on the emergence of USUV in Austria, which was probably introduced by migrating birds, showed that in the summer of 2001 several bird species were infected with USUV, and the epidemic, especially in blackbirds, was stable for more than one summer in a central European region.[2]

Little is known about WNV infections in wild birds in central Europe. There are limited data on the WNV seroprevalence in passerine birds in Poland, indicating that it is at a low level.[8] A study of WNV infections in Austria did not detect WNV in dead wild birds, and the investigators concluded that WNV has no measurable impact in Austria.[20] However, in the United Kingdom serologic evidence for WNV and USUV was reported in wild resident and migrating birds, as well as in sentinel chickens.[21] Detection of the WNV genome was demonstrated in crows.[10] In our study, none of 41 *Corvidae* samples showed evidence of an antibody response to WNV.

Infections with WNV in migratory birds in Europe can occur either through exposure in Europe, on migratory routes, or at wintering sites in Africa. The sporadic outbreaks of WNV infections in areas in southern Europe such as Romania, Tuscany in Italy, and Camargue in France do not support the assumption that WNV is endemic in these regions but may indicate that WNV is occasionally imported to these areas. In tracking the migratory route of birds from Europe to Africa, it is noteworthy that there are two major destinations, one to western regions of Africa and the other to eastern parts of Africa. On the route via southeastern Europe it was observed that WNV epidemics occur in countries such as Romania and Israel.[22–24] Many bird species rest in Israel before or after the flight across the desert during autumn and spring migration.[6] Sporadic introduction of WNV by migrating birds to central Europe may therefore be possible.

However, there are limited data on the viremic phase in WNV-infected birds. In experimental infections of different bird species, viremia in birds was high for approximately four days to enable infection of mosquitoes.[25] It is doubtful whether this period is long enough for direct import of the virus from disease-endemic areas in Africa to central Europe. However, infections with USUV in Austria support the assumption that flavivirus infections might be imported and maintained in temperate regions.[2]

It remains unclear why there is a low level percentage of antibodies to WNV in European migrating birds without clear evidence of WNV-diseased birds. In contrast to birds in the United States showing clinical symptoms, European birds have long been exposed to WNV on their migration routes to and from Africa. Over centuries, this exposure might have induced a natural resistance to WNV infections in European birds, whereas in America WNV was introduced into a highly susceptible bird population that had never been exposed to this virus.

There is evidence of only a few human WNV infections imported from the United States into Germany. Because of climate warming, it must be assumed that further WNV infections limited in time and region might appear, similar to the current situation in southeastern Europe.

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