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► **To cite this version:**

Tanja Meister, Helga Lussy, Tamás Bakonyi, Silvie Šikutová, Ivo Rudolf, et al.. Serological evidence of continuing high () activity and establishment of herd immunity in wild birds in Austria. *Veterinary Microbiology*, 2008, 127 (3-4), pp.237. <10.1016/j.vetmic.2007.08.023>. <hal-00532311>

HAL Id: hal-00532311

<https://hal.science/hal-00532311v1>

Submitted on 4 Nov 2010

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HAL Authorization

Accepted Manuscript

Title: Serological evidence of continuing high *Usutu virus* (*Flaviviridae*) activity and establishment of herd immunity in wild birds in Austria

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PII: S0378-1135(07)00415-4
DOI: doi:10.1016/j.vetmic.2007.08.023
Reference: VETMIC 3806

To appear in: *VETMIC*

Received date: 2-7-2007
Revised date: 14-8-2007
Accepted date: 15-8-2007

Please cite this article as: Meister, T., Lussy, H., Bakonyi, T., Šikutová, S., Rudolf, I., Vogl, W., Winkler, H., Frey, H., Hubálek, Z., Nowotny, N., Weissenböck, H., Serological evidence of continuing high *Usutu virus* (*Flaviviridae*) activity and establishment of herd immunity in wild birds in Austria, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.08.023

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2 **Serological evidence of continuing high *Usutu virus (Flaviviridae)* activity and**
3 **establishment of herd immunity in wild birds in Austria**
4

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1 **Abstract**

2 *Usutu virus* (USUV), family *Flaviviridae*, has been responsible for avian mortality in Austria
3 from 2001 - 2006. The proportion of USUV-positive individuals among the investigated dead
4 birds decreased dramatically after 2004. To test the hypothesis that establishment of herd
5 immunity might be responsible, serological examinations of susceptible wild birds were
6 performed.

7 Blood samples of 442 wild birds of 55 species were collected in four consecutive years (2003
8 - 2006). In addition, 86 individuals from a birds of prey rehabilitation centre were bled before,
9 at the peak, and after the 2005 USUV transmission season in order to identify titre dynamics
10 and seroconversions. The haemagglutination inhibition test was used for screening and the
11 plaque reduction neutralization test for confirmation. While in the years 2003 - 2004 the
12 proportion of seropositive wild birds was <10%, the percentage of seroreactors raised to
13 >50% in 2005 and 2006. At the birds of prey centre, almost three quarters of the owls and
14 raptors exhibited antibodies before the 2005 transmission season; this percentage dropped to
15 less than half at the peak of USUV transmission and raised again to almost two thirds after the
16 transmission season.

17 These data show a from year to year continuously increasing proportion of seropositive wild
18 birds. The owl and raptor data indicate significant viral exposure in the previous season(s),
19 but also a number of new infections during the current season, despite the presence of
20 antibodies in some of these birds. Herd immunity is a possible explanation for the significant
21 decrease in USUV-associated bird mortalities in Austria during the recent years.

22

23 *Keywords:* Usutu virus, USUV, serology, wild birds, herd immunity

24

1 **1. Introduction**

2 *Usutu virus* (USUV), a member of the Japanese encephalitis virus (JEV) antigenic
3 group within the mosquito-borne cluster of the genus *Flavivirus* (Kuno et al., 1998) was
4 isolated for the first time from mosquitoes (*Culex univittatus*) in South Africa in 1959 and
5 named after a river in Swaziland. Although the virus had been detected several times in
6 different mosquito and bird species in Africa, it had never been associated with clinical
7 disease in birds or mammals and was therefore widely scientifically ignored. In summer 2001,
8 however, USUV emerged unexpectedly in central Europe and was responsible for an episode
9 of mortality among Eurasian blackbirds (*Turdus merula*) and great grey owls (*Strix nebulosa*)
10 in and around Vienna, Austria (Weissenböck et al., 2002). In the following years the same
11 virus strain continued to kill birds in eastern Austria (Weissenböck et al., 2003b; Chvala et al.,
12 2007). This observation showed that USUV had managed to overwinter and had been able to
13 establish an efficient local bird-mosquito transmission cycle (Weissenböck et al., 2003a).
14 Meanwhile, USUV-associated bird mortality has been registered in other central European
15 countries like Hungary (Bakonyi et al., unpublished data), Switzerland (ProMED-mail) and
16 Italy (Dorrestein et al., 2007). Surveillance data of USUV-associated bird deaths in Austria
17 indicated that seasons of massive USUV-associated bird losses (2001-2003) were followed by
18 seasons with significant decline of USUV-linked avian mortality (2004-2006) (Chvala et al.,
19 2007). In addition to climatic reasons (the summers of 2004 and 2005 had unusually low
20 average temperatures in Austria, <http://www.zamg.ac.at>) or decreased virulence of the
21 circulating virus another possible explanation for this phenomenon would be a progressive
22 seroconversion in the Austrian wild bird population.

23 As it has to be expected that USUV will continue to expand its area of activity during
24 the next years, data on seroprevalence and potential herd immunity in the European area

1 affected first, i.e. eastern Austria, might be useful for other scientists and wildlife
2 conservationists having to deal with this phenomenon in the future.

3 The aims of the present study were first to evaluate the proportion of USUV antibody
4 positives among wild birds in Austria and to record changes during the course of time.
5 Second, we intended a longitudinal serological study with three blood collection timepoints
6 from the same individuals during one transmission season in order to determine the dynamics
7 of change in antibody titre to USUV in naturally infected birds. For this part of the study an
8 owl and raptor rehabilitation centre situated within the USUV-endemic area in eastern Austria
9 was chosen because i) some owl species (great grey owl, *Strix nebulosa*, Tengmalm's owl,
10 *Aegolius funereus*) easily acquired USUV infection and also succumbed to it, ii) birds of prey
11 and owls were found to be frequently affected by the related *West Nile virus* (WNV) in North
12 America (Fitzgerald et al., 2003; Gancz et al., 2004; Wünschmann et al., 2004) and iii)
13 because the centre offered a large collection of wild birds in an open mosquito-accessible
14 environment with the opportunity of repeated blood collections of the same birds, something
15 not easily done with wild birds.

16

17 **2. Materials and Methods**

18

19 ***2.1. Sera for seroprevalence study***

20 Bird sera were collected in four consecutive years, between August 2003 and May
21 2006. As the transmission season of USUV is most likely restricted to the months July to
22 September, the data of the 2006 sera reflect viral exposure which had happened up to the
23 2005 transmission season. In total, sera of 442 birds were included. A total of 113 sera were
24 collected in 2003 (between August and December), 109 sera in 2004 (January to October),
25 197 sera in 2005 (March to October), and finally, 23 sera were collected in the first five

1 months of 2006. As it significantly influences the interpretation of the results, a possible
2 exposure in the previous year(s) was especially considered for the 2005 and 2006 sera. The
3 sources of sera were i) wild birds captured in mist nets or other trapping devices especially for
4 the purpose of USUV serosurveillance (2003: 14; 2004: 27; 2005: 91; 2006: 23), ii) sick or
5 injured birds brought to the bird clinic of the University of Veterinary Medicine, Vienna, for
6 treatment (2003: 28; 2004: 2; 2005: 45), iii) birds from the above mentioned owl and raptor
7 rehabilitation centre (2003: 28; 2005: 38), and iv) dead birds submitted for necropsy (2003:
8 43; 2004: 80; 2005: 23). The sera originated from 55 different species of birds. The huge
9 majority of the birds were from USUV-endemic areas in Vienna, Lower Austria and
10 Burgenland. Only seven birds were from areas where USUV activity has not been found so
11 far.

13 **2.2. Longitudinal serosurvey in captive birds of prey**

14 All birds originated from a birds of prey rehabilitation centre which is located in the
15 village Haringsee (48°11' N, 16°46' E) in the geographic area Marchfeld in Lower Austria.
16 The entire area is 11.000 m² in size. There are 70 separate aviaries covering a total of 3.000
17 m². The birds were separated according to species, and aviaries with birds of the same species
18 were located in close proximity to each other. The station mainly provides medical care and
19 shelter for injured bird foundlings and confiscated animals, and information for the interested
20 public. USUV activity has been recognized in the area since 2003 with the virus found in
21 dead blackbirds and in mosquitoes (Chvala et al., 2007).

22 Blood samples were collected from 86 birds belonging to nine species: six species of
23 the family *Strigidae*: eight eagle owls (*Bubo bubo*), 18 barn owls (*Tyto alba*), 14 tawny owls
24 (*Strix aluco*), four little owls (*Athene noctua*), five long-eared owls (*Asio otus*), one Ural owl
25 (*Strix uralensis*), two accipitrid species: 20 common buzzards (*Buteo buteo*) and four marsh

1 harriers (*Circus aeruginosus*), and one falcon species, namely 12 common kestrels (*Falco*
2 *tinnunculus*). From each bird three blood samples were taken at approximately two-month
3 intervals during 2005: the first blood samples prior to any anticipated USUV activity (May
4 25), the second sample on August 29 at the time when in the previous years USUV activity
5 had reached its peak, and the final sample was taken October 17, 2005, when, according to the
6 experiences from the previous years, USUV activity should have ceased and antibodies due to
7 recent exposure should have developed. All birds were after hatch-year birds (older than 1
8 year), except for one Ural owl, which was a hatch-year fledgling. None of the birds showed
9 clinical signs during the surveillance period. For USUV antibody assays 0.2 – 0.5 ml of blood
10 was drawn from the cutaneous ulnar vein. The blood was transferred into heparin-lithium
11 tubes (Sarstedt, Nürnberg, Germany) and centrifuged at 2000 x g for 15 min. The plasma
12 was separated from the clot and stored at -20 °C until use. In order to rule out test
13 variabilities, all three blood collections of the birds of prey rehabilitation centre were tested in
14 one investigation and carried out and read by the same investigator.

15

16 **2.3. Serological tests**

17 The majority of the bird sera obtained for the seroprevalence study were examined by
18 the haemagglutination inhibition test (HIT). Whenever possible, HIT positives were
19 confirmed by the plaque reduction neutralization test (PRNT). However, due to the small
20 quantity of some sera, either this confirmation could not be performed or it was decided to use
21 the PRNT only.

22 All serum samples of the longitudinal study were analysed by HIT for initial
23 screening. Positive samples (titre $\geq 1:20$) were also tested by PRNT to evaluate the specificity
24 of the HIT. To rule out a possible cross reaction of the tests with *tick-borne encephalitis virus*
25 (TBEV) and WNV a number of randomly selected USUV-positive sera (TBEV: 55; WNV:

1 49) were also tested with serological test systems established for detection of antibodies to
2 these viruses.

3

4 **2.4. HIT for USUV and TBEV antibodies**

5 The standard HIT was performed as previously described by Clarke and Casals (1958)
6 and as adapted for USUV by Chvala et al. (2005). In brief, non-specific inhibitors and natural
7 haemagglutinins were removed by kaolin treatment and absorption with goose erythrocytes,
8 respectively. Serial dilutions of kaolin-treated bird sera were mixed with 8 haemagglutinating
9 (HA) units of USUV strain Vienna 2001-blackbird or TBEV strain KEM₁ antigen (Molnar,
10 1982), respectively. Tests were performed in U-shaped microtitre plates. The HIT titre was
11 determined as the highest serum dilution that caused complete inhibition of erythrocyte
12 agglutination. Sera with a titre of 1:20 and higher were considered positive.

13

14 **2.5. PRNT for USUV and WNV antibodies**

15 The PRNT method for USUV and WNV antibodies was performed as described by de
16 Madrid and Porterfield (1974), adopted to a microtechnique (Hubálek et al., 1979).
17 The sera were inactivated at 56 °C for 30 min prior to testing. The PRNTs were run in
18 microtitre plates with flat-bottomed wells. For USUV the above mentioned virus strain
19 Vienna-2001 blackbird (Bakonyi et al., 2004) and porcine kidney (PK) cells, and for WNV
20 the WNV topotype strain Eg-101 and the pig kidney embryo cell line SPEV was used.
21 Twofold serum dilutions were made in Minimal Essential Medium (MEM), or in case of
22 WNV in L-15 medium; 30 µl of diluted sera were mixed with 30 µl of virus suspension
23 containing 100 plaque-forming units of the virus and incubated for 60 min at 37 °C. Then 60
24 µl of cell suspension in MEM with 3% foetal calf serum (in case of WNV L-15 medium with
25 2% foetal calf serum) was added to each well and incubated at 37 °C for 4 h. Thereafter 120

1 μl of a carboxy-methyl cellulose overlay was added to each well and incubated at 37 °C for 3
2 days (5 days in case of WNV). The fluid was removed and 150 μl of the colouring naphthol
3 blue black solution was added for 40 min at room temperature. The PRNT titre was
4 determined as the highest serum dilution with a 90% reduction of the number of plaques. Sera
5 with a titre of at least 1:20 were considered positive. The specificity of this assay for
6 antibodies to the viruses tested (i.e. USUV and WNV) had been validated by using WNV-
7 and USUV-positive test sera. Cross-reactivity was minimal and only occurred in sera with
8 high titres to one of the viruses to a titre of at least 4 dilution steps less than the homolog
9 virus.

10

11 ***2.6. RT-PCR for detection of viraemia***

12 At the assumed peak of USUV activity (August), we also took blood samples from 32
13 larger birds (eight eagle owls, 20 buzzards, and four marsh harriers) for determination of
14 viraemia. From these birds, blood was drawn from the ulnar vein into EDTA-treated tubes
15 (Sarstedt, Nürnberg, Germany), centrifuged at 6700 x g for 5 min, and the plasma was saved
16 for serological studies. Peripheral blood mononuclear cells (PBMCs) were purified from the
17 buffy coat using Erythrocyte Lysis buffer (Qiagen, Hilden, Germany) according to the
18 manufacturer's instructions. RNA was extracted from the PBMCs using the QiaAmp Viral
19 RNA Mini Kit, and RT-PCRs were performed in a continuous one-step RT-PCR system
20 employing USUV-specific primer pairs (Bakonyi et al., 2004, Weissenböck et al., 2004).

21

22 **3. Results**

23

24 ***3.1. Antibodies to USUV are found in an increasing proportion of wild birds between 2003*** 25 ***and 2006***

1
2 Of the 222 birds tested in 2003 and 2004 19 (8.5 %) were positive for USUV by HIT.
3 The titres ranged from 1: 20 to 1:1280, with a geometrical mean titre of 51.8. All positives
4 except one were confirmed by PRNT. Four of the positive birds were necropsy cases with an
5 acute USUV infection. Among the 19 examined owls six (31.6 %) were positive. The USUV
6 positive sera were also tested by HIT for antibodies to TBEV. One serum (with an USUV titre
7 of 1:1280) showed a positive reaction (1:80). All other sera were TBEV antibody negative.
8 In 2005 and early 2006 a total of 220 sera was tested by HIT (150) and/or PRNT (157). In 87
9 cases a comparative evaluation of both tests could be performed. In these years 119 (54%) of
10 the samples were found positive: eleven exclusively by HIT (no PRNT performed), 29
11 exclusively by PRNT (no HIT performed), 68 with correspondingly positive HIT and PRNT
12 results, and eleven cases with positive HIT and negative (7) or not analysable (4) PRNT.
13 These results are compiled in Table 1 and Fig. 1. Seventy-one (43 of which were positive) of
14 the 2005 samples and all 23 (nine of which were positive) 2006 samples were taken before
15 July, i.e. before the actual year's transmission season (Table 2). Thus these samples indicate
16 antibody titres acquired the years before. Of the sick or dead birds examined 29 had an acute
17 USUV infection with characteristic lesions and presence of virus in a number of tissues. Out
18 of these birds only four were serologically positive.

19 An interesting aspect of this study were the serological data of the 78 examined
20 juvenile birds (Table 2). Forty-two (54.5%) of them were serologically USUV antibody
21 positive. Among them were five Ural owls whose antibody titres were 1:20 (1), 1:40 (2), and
22 1:80 (2). The adult females that produced these six nestlings had titres of 1:320 and 1:2560,
23 respectively. The mother of the other juveniles was unknown.

24

1 ***3.2. Captive birds of prey show a high proportion of USUV antibody positives and***
2 ***considerable HIT titre dynamics during one transmission season***

3 In May 2005, 63 (73.3 %) out of 86 birds exhibited HIT antibodies to USUV (titres
4 $\geq 1:20$). The titres ranged from 1:20 to 1:640, with the majority (69.8 %) having a titre of 1:80
5 or lower. In August 2005, the number of seropositives declined to 39 (45.3 %), the majority of
6 which (56.4 %) had low titres of 1:20 or 1:40. In October 2005, 56 (65.1%) were serologically
7 positive, with a higher proportion of medium and high titres (almost 60.7% with titres $\geq 1:80$)
8 compared to the previous two timepoints (Figs. 2 - 3).

9 A total of 143 sera, which showed a HIT titre of least 1:20 were tested by PRNT for
10 confirmation. 85.3% of the PRNT titres were in accordance with the HIT results. Sixty-two of
11 the sera sampled in May were tested by PRNT. Of these, 25 showed a lower titre compared to
12 HIT, four HIT positives were negative by PRNT, and one PRNT titre could not be analyzed
13 due to cytotoxicity of the serum. Of the sera taken in August 34 were tested by PRNT. In ten
14 of the samples the PRNT titre was lower than the HIT titre. Two sera were negative by PRNT
15 and ten were cytotoxic. Of the October samples, 47 were tested by PRNT. Twenty-five sera
16 had a lower PRNT titre compared to HIT. Five sera were negative and six were cytotoxic.

17

18 ***3.4. Low titre haemagglutinating antibodies to TBEV and neutralizing antibodies to WNV***
19 ***are present in a few birds***

20 A portion of USUV antibody positive sera from the third bleeding time were also
21 tested by HIT for TBEV antibodies. Only seven of 55 exhibited a low-range titre of 1:20 and
22 1:40, respectively.

23 Forty-nine USUV antibody positive birds were tested by PRNT for WNV antibodies.
24 Of 19 birds from the first bleeding time, 15 were negative, one kestrel showed a titre of 1:40,
25 and two marsh harriers and one barn owl had titres of 1: 20. Of the eleven tested birds of the

1 second bleeding time, seven were negative, two birds exhibited titres of 1:20 (common
2 buzzard, Ural owl) and two kestrels had titres of 1:80 and 1:160, respectively. In October, the
3 third bleeding, 19 birds were tested, 12 of which were negative; five had titres of 1:20, and
4 two, both kestrels, showed titres of 1:80 and 1:160, respectively.

5

6 ***3.5. No evidence of viraemia in the sampled birds at the peak of the transmission period***

7 USUV nucleic acid sequences were not detected in any of the examined PBMC
8 samples by RT-PCR.

9

10 **4. Discussion**

11

12 Since its first documented emergence in central Europe in 2001, USUV has been
13 associated with rising avian mortality in the affected areas which was followed by a rapid
14 decline of USUV-associated deaths by 2004 until present. A major aim of the study was to
15 discern, whether an increasing number of seroreactors in the wild bird population might have
16 contributed to this phenomenon. The data point towards a low USUV antibody prevalence in
17 samples from 2003 and 2004, and a clearly increased antibody prevalence in samples taken in
18 2005 and 2006. This change is not likely to be due to biased sample selection. Especially two
19 subpopulations of examined birds – blackbirds and owls – originated from comparable
20 habitats during the entire investigation period.

21 In the cases in which comparative investigations of sera were carried out by HIT and
22 PRNT the majority of the HIT titres were confirmed by PRNT. Generally the PRNT titres
23 were lower. Although the HIT is not considered to be highly specific, it proved useful as
24 initial screening test in the present study. Possible cross-reactions or false positive reactions
25 did not occur on a grand scale. The only other flavivirus known to be enzootic in Austria is

1 TBEV. The most likely explanation for the few seroreactors to TBEV in the used HIT is
2 cross-reactivity with USUV, as the TBEV titres were generally 8-16 times lower than those to
3 USUV. HIT cross reactivity between these two distantly related flaviviruses has also been
4 previously noticed (Casals and Brown, 1954, De Madrid and Porterfield, 1974, Stiasny et al.,
5 2006). Also cross reactivity of USUV with WNV including associated lineages (e.g.
6 *Rabensburg virus* (RabV) (Bakonyi et al., 2005)) is very likely. Using the less specific HIT,
7 distinction of USUV- and WNV-titres might have been difficult or impossible. Therefore, the
8 more specific PRNT was used in the search for WNV antibodies. The WNV serological data
9 of a randomly chosen subset of samples showed several reactors, the majority of which had a
10 low titre. These low titres are explainable by cross-reactivity to USUV, as all these cases had
11 high USUV titres. The few birds with a moderate or high titre to WNV (e.g. common kestrel)
12 could represent WNV- (or RabV-) infected animals, because the locality, where RabV was
13 isolated, is situated very closely to the USUV study site (Hubálek et al., 1998). As the vast
14 majority of these birds had high haemagglutinating and moderate neutralizing antibody titres
15 to USUV, cross-reactivity of WNV antibodies in the USUV assays seem rather unlikely.
16 Thus, these kestrels might represent double infections with USUV and a representative of one
17 of the WNV lineages. The presence of a few seroreactors to WNV is not surprising and is in
18 line with previous seroepidemiological studies from comparable geographical regions
19 (Hubálek and Halouzka, 1999, Hubálek et al., 2005).

20 The serological data indicate that species do not differ in the likelihood to acquire
21 USUV infection. However, there seem to be great differences with respect to the expression
22 of clinical symptoms: While certain species, like blackbirds, great grey owls and obviously
23 house sparrows – as recently shown in Switzerland (Steinmetz et al., 2007) - succumb in high
24 numbers to the infection, other species never exhibited significant USUV-associated
25 mortality.

1 A small number of seropositive birds, especially among those captured in 2005, were
2 long distance migrants, i.e. birds with wintering habitats in sub-Saharan Africa. Adult birds of
3 this group could well have acquired USUV antibodies in Africa. However, the vast majority
4 of these birds were identified as juveniles, i.e. they had hatched in Austria several weeks or
5 months prior to sampling, and provided that maternally transferred antibodies do not last until
6 several months of age, they most likely have been exposed to the virus in Austria. Data
7 concerning persistence of maternally transferred antibodies in wild birds are scarce (Müller et
8 al., 2004, Hahn et al., 2006); thus it cannot be definitely excluded that some of these
9 antibodies have their origin in Africa. The few seropositive juvenile birds for which the
10 mother was known were five Ural owl nestlings with an age of 62 days at sampling. The
11 USUV antibody levels of these birds were markedly lower than those of their mothers. As
12 sampling in these nestlings took place before the transmission season the results suggest that
13 they might have acquired antibodies through passive transmission and that detectable amounts
14 of passively transferred USUV antibodies are detectable up to two months. In contrast, Gibbs
15 et al. (2005) found maternal WNV antibodies in rock pigeons only up to 30 days after
16 hatching. Alternatively, it cannot be ruled out that the juvenile Ural owls were exposed to one
17 of the alternative transmission routes (see below), which are not necessarily linked with
18 mosquito activity.

19 While in 2003 the proportion of USUV-positives among dead birds collected during a
20 surveillance program was more than 50 %, this percentage dropped to 5 % and less in 2004
21 and 2005 (Chvala et al., 2007). One possible explanation for such a phenomenon could be
22 establishment of herd immunity resulting in an increasing number of birds born with passive
23 immunity under the protection of which active immunity can develop in the case of exposure.
24 Although the serological data of the 2004 birds did not yet suggest such a phenomenon, the
25 closer inspection of the 2005 data shows that more than a third of the samples were taken

1 before the transmission season and thus indicate titres acquired in the previous year(s) or
2 through maternal antibodies in hatchlings. In fact, 60 % of this subset were positive which
3 indicates that already in (late) 2004 many more birds were exposed to the virus and
4 subsequently seroconverted than the samples taken in 2004 suggest. From this point of view it
5 becomes evident that in parallel with the significant decline of USUV-associated avian
6 mortality the number of seropositive birds in the endemic areas increases steadily. Therefore
7 it is a likely possibility that a rather rapid establishment of herd immunity has been
8 responsible for apparent disappearance of USUV-associated bird deaths, despite continuing
9 viral circulation. The high percentage of seropositives to a circulating arbovirus with a bird-
10 mosquito transmission cycle is unparalleled in other endemic transmission cycles so far.
11 Seroprevalence rates of WNV, *Saint Louis Encephalitis virus*, and *Sindbis virus* usually only
12 reach 1,5-9,7 % (McLean et al., 1988, Antipa et al., 1984, Juricova et al., 1987, Juricova et al.,
13 1989, Beveroth et al., 2006). The only other paper which claims a similar high transmission
14 rate, however using the more sensitive 50% PRNT (compared to the 90% PRNT used in the
15 present study), does not only suggest local transmission but also continuous introduction of
16 virus by migratory birds to the British Isles (Buckley et al., 2003). In the case of USUV,
17 however, one genetically stable virus strain established a local transmission cycle in local
18 birds and mosquitoes in Austria with a tendency of slow but steady spread to adjacent areas
19 (Chvala et al., 2007).

20 In addition to the indisputable increase of seroreactors within the wild bird population
21 also other factors could have contributed to the rapid decline of USUV-associated avian
22 deaths registered during a 3-year period of dead bird surveillance (Chvala et al., 2007). On the
23 one hand climate factors could have been influential, on the other hand decreased virulence of
24 the circulating USUV strain could also have played a role. Data from other flaviviruses (e.g.
25 WNV) showed that virulence for certain bird species is strain-dependent (Brault et al., 2004)

1 and it has been suggested that especially mutations in certain E-protein gene regions resulting
2 in loss of glycosylation were responsible for reduced virulence or neuroinvasiveness (Beasley
3 et al., 2005). For USUV, currently no complete sequences or experimental data of virus
4 strains isolated in different years are available. However, sequencing of 88 % of the E coding
5 region of 12 USUV isolates from 3 consecutive years (2003-2005) revealed only single
6 random mutations, all of which except one did not result in amino acid changes (Chvala et al.,
7 2007).

8 The fact that already in 2003 the proportion of seropositives among the surveyed owl
9 species tawny owl and long eared owl was significantly above the average prompted us to
10 undertake a more thorough investigation among the birds in this rehabilitation centre. The
11 overall seroprevalence among these birds almost doubled after two years. We expected new
12 insights into the infection dynamics of USUV infections from the comparative examination of
13 three blood samples per bird taken at three different timepoints during one transmission
14 season. Already in May, well before the start of the transmission season, a high percentage of
15 the blood samples exhibited antibodies to USUV. This observation correlates well with the
16 generally high seroprevalence in the wild bird population, indicating again viral exposure in
17 the previous season(s). Transmission of mosquito-borne flaviviruses occurs predominantly
18 from viraemic birds to mosquitoes which after completion of the extrinsic incubation period
19 are capable of transmitting the virus to a new avian host. Under natural conditions this is
20 certainly the most efficient and most common transmission route. In more artificial settings,
21 such as the case for caged wild birds, also other modes of flaviviral transmission have been
22 observed. WNV, for example, can also be transmitted by direct contact (Komar et al., 2003),
23 by eating infected reservoir hosts (Austgen et al., 2004, Nemeth et al., 2006) and especially in
24 owls, it has been speculated that louse flies might serve as additional vectors (Gancz et al.,
25 2004). Many of the owls of the present study were infested with louse flies, too, and they

1 probably might have contributed to the viral distribution among the birds within certain
2 aviaries. However, there is no formal proof as yet that louse flies are competent vectors for
3 flaviviruses. These transmission modes are not restricted to seasons of mosquito activity and
4 could theoretically have occurred within this bird collection at any time of the year.

5 During the following six-month observation period some interesting changes in titre
6 development were noticed. From the first to the second bleeding the geometric mean titre of
7 most bird species markedly dropped as did the total number of seropositives. This can be
8 explained by a natural decline of antibody titres during a period without viral activity. In
9 several birds the titre decline within this rather short time interval was intriguingly
10 pronounced. This observation suggests that even after natural infection flaviviral titres in
11 birds are generally not very robust and long lasting, but subject to considerable variations
12 within short times and it can certainly not be assumed that such antibodies persist life-long.

13 After the transmission season, which - based on dead bird surveillance data - ends in mid-
14 September, seroconversions were noted in several birds. Some had not had any detectable
15 antibodies before and some had had low titres. In several birds the serotitres continued to drop
16 until the last bleeding which might either indicate lack of exposure or protective titres
17 preventing infection and viral replication. However, despite the fact that seroconversions
18 obviously occurred, by RT-PCR of PBMCs of selected birds taken during the transmission
19 season no evidence of viraemia was found. Taking into account that viraemia in flavivirus
20 infections of birds is usually short-lived, i.e. not longer than a few days (Nemeth et al., 2006)
21 it simply seems to have been bad luck that no viraemic bird had been detected by examining a
22 single blood sample during the entire transmission season. Taking all data together, the
23 number of seropositives had risen between the second and third bleeding and the proportion
24 of medium and high titres was highest at the last bleeding. These data clearly indicate that
25 despite a high pre-existing herd immunity viral activity still leads to new infections and

1 seroconversions. This fact that flaviviral circulation despite the presence of significant
2 immunity is easily possible is a significant observation which is especially important for the
3 understanding of concepts of flavivirus epidemiology. This study also clearly shows the lack
4 of pathogenicity of USUV for the particular species of owls and birds of prey kept in
5 captivity. Since the first detection of viral activity in the area in 2003, no diseases or deaths of
6 birds which could be attributed to USUV infection were noticed in this particular region. This
7 observation is in sharp contrast to the documented vulnerability of one owl species (great grey
8 owl) (Weissenböck et al., 2002) with its natural habitat in periarctic zones. This species has
9 also proved to be highly vulnerable to infection with the related WNV (Gancz et al., 2004).

10 In conclusion, the findings presented in this paper suggest that USUV circulates very
11 efficiently between local birds and mosquitoes in eastern Austria. After a few years of
12 presence with an initial severe bird mortality the virus produced a high seroprevalence in the
13 susceptible hosts which seems to be sufficient for establishment of an (at least currently)
14 stable herd immunity.

16 ACKNOWLEDGEMENTS

17 This study was funded by a grant from the Austrian Federal Ministry for Health and
18 Women's Issues, the grant OTKA D048647, and partially supported by the Grant Agency of
19 the Czech Academy of Sciences (IAA600930611).

20 We thank Christiane Bukovsky, Sonja Chvala, Thomas Filip, Christine Noestler,
21 Christine Truxa, Franziska Resch and the colleagues from the Clinic for Avian, Reptile and
22 Fish Medicine for their contributions in sample collection, and to Jiri Halouzka for his help
23 with treatment of avian sera. The phlebotomy procedures have been approved by the Austrian
24 Committee for Animal Trials (GZ 68.205/95-BrGT/2004). The help of Gerhard Loupal with
25 ornithological questions is gratefully acknowledged.

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1 **Figure Captions**

2 **Fig. 1.** Histogram showing the ratio of serologically USUV-positive and USUV-negative
3 (based on both HIT and PRNT data) birds among the animals examined from 2003 to 2006.
4 Only bird species of which more than 7 individuals were examined are included. 295
5 examined birds (66.7% of the total) are presented in this figure.

6 Legend: EB: Eurasian blackbird, TO: Tawny owl, LO: Long-eared owl, ED: Eurasian
7 collared dove, TS: Tree sparrow, GT: Great tit, ER: European robin, GW: Great spotted
8 woodpecker, BC: Blackcap, UO: Ural owl

9
10 **Fig. 2.** Histogram depicting the percentage of captive birds of prey with certain HIT antibody
11 titres at the different timepoints of sampling.

12
13 **Fig. 3.** Histogram depicting the HI titre dynamics in the birds of prey during timecourse. For
14 each species which comprised more than 7 individuals the geometric mean titre, and the
15 minimum and maximum titres are shown in columns. The three columns for each bird species
16 demonstrate the values at the 3 sampling timepoints.

17

Table 1. Compilation of all wild birds, sorted according to numbers, species and years, which were subjected to serological investigation. Positive means titres $\geq 1:20$ to USUV, either with HIT or PRNT. S = short distance migrant (winter habitat: mediterranean); L = long distance migrant (winter habitat: sub-saharan Africa); W = winter guest

No	Common name	Scientific name	Total	2003	2004	2005	2006	All years
				positive/total	positive/total	positive/total	positive/total	positive/total
1	Eurasian blackbird	<i>Turdus merula</i>	165	3/33	8/83	20/35	6/14	37/165
2	Blackcap	<i>Sylvia atricapilla</i>	S 23			12/23		12/23
3	Ural owl	<i>Strix uralensis</i>	22			16/22		16/22
4	Eurasian collared dove	<i>Streptopelia decaocto</i>	20	0/11		6/9		6/20
5	Great tit	<i>Parus major</i>	19	0/1	0/9	3/9		3/19
6	Long-eared owl	<i>Asio otus</i>	17	3/11		5/6		8/17
7	Great spotted woodpecker	<i>Dendrocopos major</i>	16			2/7	3/9	5/16
8	Kestrel	<i>Falco tinnunculus</i>	12	0/7		4/5		4/12
9	European robin	<i>Erithacus rubecula</i>	S 11			5/11		5/11
10	Tawny owl	<i>Strix aluco</i>	10	3/8	0/1	1/1		4/10
11	Jackdaw	<i>Corvus monedula</i>	10	0/3		6/7		6/10
12	Song thrush	<i>Turdus philomelos</i>	S 9	0/4	0/1	4/4		4/9
13	Tree sparrow	<i>Passer montanus</i>	9	0/8		0/1		0/9
14	Jaybird	<i>Garrulus glandarius</i>	8	1/2	1/4	2/2		4/8
15	Bearded vulture	<i>Gypaetus barbatus</i>	7	0/2		2/5		2/7
16	Blue tit	<i>Parus caeruleus</i>	7	0/1	0/6			0/7
17	Reed warbler	<i>Acrocephalus scirpaceus</i>	L 7			3/7		3/7
18	Common buzzard	<i>Buteo buteo</i>	6	0/2	0/2	0/2		0/6
19	Hooded crow	<i>Corvus corone cornix</i>	6	0/2		3/4		3/6
20	Rook	<i>Corvus frugilegus</i>	W 5	0/3	0/2			0/5
21	Nuthatch	<i>Sitta europaea</i>	4			1/4		1/4
22	Eagle owl	<i>Bubo bubo</i>	3			1/3		1/3
23	Marsh harrier	<i>Circus aeruginosus</i>	L 3	0/2		1/1		1/3
24	Yellowhammer	<i>Emberiza citrinella</i>	3	0/3				0/3
25	Barn-swallow	<i>Hirundo rustica</i>	L 2			2/2		2/2
26	European goldfinch	<i>Carduelis carduelis</i>	2	0/2				0/2
27	Kingfisher	<i>Alcedo atthis</i>	2	0/2				0/2
28	Lesser whitethroat	<i>Sylvia curruca</i>	L 2			1/2		1/2
29	Middle-spotted woodpecker	<i>Dendrocopos medius</i>	2			0/2		0/2
30	Mute swan	<i>Cygnus olor</i>	2			0/2		0/2
31	Pheasant	<i>Phasianus colchicus</i>	2	0/1		1/1		1/2
32	Reed bunting	<i>Emberiza schoeniclus</i>	S 2			1/2		1/2
33	Whitethroat	<i>Sylvia communis</i>	L 2			1/2		1/2
34	Barn owl	<i>Tyto alba</i>	1			1/1		1/1
35	Black redstart	<i>Phoenicurus ochruros</i>	S 1			1/1		1/1
36	Brambling	<i>Fringilla montifringilla</i>	W 1			0/1		0/1
37	Capercaillie	<i>Tetrao urogallus</i>	1			0/1		0/1
38	Chaffinch	<i>Fringilla coelebs</i>	1		0/1			0/1
39	Chiffchaff	<i>Phylloscopus collybita</i>	S 1			0/1		0/1
40	Crossbill	<i>Loxia curvirostra</i>	1			0/1		0/1
41	Garden warbler	<i>Sylvia borin</i>	L 1			1/1		1/1
42	Greenfinch	<i>Carduelis chloris</i>	1	0/1				0/1
43	House martin	<i>Delichon urbica</i>	L 1			1/1		1/1
44	Mallard duck	<i>Anas platyrhynchos</i>	1	0/1				0/1
45	Nightingale	<i>Luscinia megarhynchos</i>	L 1			0/1		0/1
46	Indian peafowl	<i>Pavo cristatus</i>	1			1/1		1/1

47	Penduline tit	<i>Remiz pendulinus</i>	S	1			0/1		0/1
48	Pied flycatcher	<i>Ficedula hypoleuca</i>	L	1			1/1		1/1
49	Quail	<i>Coturnix coturnix</i>	L	1			0/1		0/1
50	Red-backed shrike	<i>Lanius collurio</i>	L	1			0/1		0/1
51	Seagull	<i>Larus sp.</i>		1	0/1				0/1
52	Sparrow hawk	<i>Accipiter nisus</i>		1	0/1				0/1
53	Starling	<i>Sturnus vulgaris</i>	S	1			1/1		1/1
54	Waxwing	<i>Bombycilla garrulus</i>	W	1			0/1		0/1
55	Woodcock	<i>Scolopax rusticola</i>	S	1	0/1				0/1
				442	10/113	9/109	110/197	9/23	138/442

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Table 2. Serologically investigated wild birds, grouped according to sampling timepoint (before/after start of USUV transmission season), age (born in the year of sampling or earlier) and presence of migratory birds among the sampled individuals.

Year	2003				2004				2005				2006			
	before July		after July		before July		after July		before July		after July		before July		after July	
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
Juveniles	0	0	1	0	0	0	0	0	5*	11*	30*	31*	0	0	0	0
Migrants	0	0	1	0	0	0	0	0	0	0	8**	8**	0	0	0	0
Total	0	0	103	10	18	2	82	7	28	43	59	67	14	9	0	0

* Juvenile seropositive birds belonged to the following species: 10 Eurasian blackbirds (*Turdus merula*), 5 blackcaps (*Sylvia atricapilla*), 5 Ural owls (*Strix uralensis*), 3 Eurasian collared doves (*Streptopelia decaocto*), 2 barn swallows (*Hirundo rustica*), 2 bearded vultures (*Gypaetus barbatus*), 2 European robins (*Erithacus rubecula*), 2 reed warblers (*Acrocephalus scirpaceus*), 1 black redstart (*Phoenicurus ochruros*), 1 garden warbler (*Sylvia borin*), 1 great tit (*Parus major*), 1 house martin (*Delichon urbica*), 1 jaybird (*Garrulus glandarius*), 1 kestrel (*Falco tinnunculus*), 1 lesser whitethroat (*Sylvia curruca*), 1 nuthatch (*Sitta europaea*), 1 pied flycatcher (*Ficedula hypoleuca*), 1 song thrush (*Turdus philomelos*), 1 whitethroat (*Sylvia communis*)

** all juveniles





