Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland-Genomic and pathologic comparison to other central European outbreaks

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Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland-Genomic and pathologic comparison to other central European outbreaks

Abstract

In late summer 2006 considerable mortality in wild and captive Passeriformes and Strigiformes was observed in Zurich, Switzerland. All animals were found in a range of 2km(2). Observed clinical signs involved depression, ruffled plumage, incoordination, seizures and peracute death. Nutritional status was generally moderate to poor in wild birds, and variable in captive animals. Necropsy showed marked splenomegaly, a mild hepatomegaly, and pulmonary hyperemia in most animals. Histopathologic lesions were very discrete and consisted mainly of neuronal necrosis, leucocytolysis in and around the brain blood vessels, and miliary liver necrosis. The diagnosis Usutu virus (USUV) infection was established by USUV-specific immunohistochemistry and reverse transcription-polymerase chain reaction. Partial nucleotide sequence comparisons revealed>99% identity between the viruses that emerged in Zurich in 2006, in Vienna in 2001, and in Budapest in 2005. Since 2008 a significantly lower mortality was observed in wild Passeriformes, but USUV infection was confirmed for the first time beyond Zurich city limits. Indoor housing and regular treatment against ectoparasites are likely to have prevented acute USUV disease in captive Strigiformes. USUV is a mosquito-borne flavivirus causing fatalities in various avian species. After the initial European outbreaks in Austria in 2001 it appears that the virus has extended its range in Central Europe and has established a transmission cycle between local bird and mosquito species. Further episodes of increased avian mortality in the forthcoming years, with impact on wild and captive bird populations, predominantly Passeriformes and Strigiformes, can be anticipated. Furthermore, the possibility of broader dispersal of USUV in Europe during the next mosquito seasons must be considered and an increased mortality in Passeriformes and Strigiformes must be expected until protective "flock immunity" is established. Collections of valuable and endangered Passeriformes and Strigiformes, especially young of the year, should therefore be housed indoors or treated against ectoparasites at acceptable intervals between July and September each year.
GENOMIC AND PATHOLOGIC COMPARISON OF USUTU VIRUS EMERGENCE IN WILD AND CAPTIVE AVIAN SPECIES AT DIFFERENT LOCATIONS IN EUROPE

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Abstract:

In late summer 2006 considerable mortality in wild and captive Passeriformes and Strigiformes was observed in Zurich, Switzerland. All animals were found in a range of 2 km². Observed clinical signs involved depression, ruffled plumage, incoordination, seizures and peracute death. Nutritional status was generally moderate to poor in wild birds, and variable in captive animals. Necropsy showed marked splenomegaly, a mild hepatomegaly, and pulmonary hyperemia in most animals. Histopathologic lesions were very discrete and consisted mainly of neuronal necrosis, leucocytolysis in and around the brain blood vessels, and miliary liver necrosis. The diagnosis of an Usutu virus (USUV) infection was confirmed by immunohistochemistry and reverse transcription polymerase chain reaction. Partial nucleotide sequence comparisons revealed >99% identity between the viruses that emerged in Zurich in 2006, in Vienna in 2001, and in Budapest in 2005. Since 2008 a significant lower mortality was observed in wild Passeriformes, but USUV infection was confirmed for the first time beyond Zurich city limits. Indoor housing and regular treatment against ectoparasites apparently prevented acute USUV disease in captive Strigiformes. Usutu virus is a mosquito-borne flavivirus causing fatalities in various avian species. After the initial European outbreaks in Austria in 2001 it appears that the virus has extended its range in Central Europe and has established a transmission cycle between local bird and mosquito species. Further episodes of increased avian mortality in the forthcoming years, with impact on wild and captive bird populations, predominantly Passeriformes and Strigiformes, can be anticipated. Furthermore, the possibility of the spread of USUV through whole Europe during the next mosquito seasons must be considered and an increased mortality in Passeriformes and Strigiformes must be expected until protective “flock immunity” is established. Collections of valuable and endangered Passeriformes and Strigiformes, especially
young of the year, should be housed indoors or treated at an acceptable interval against ectoparasites between July and September each year.

**Key words:** USUV, Passeriformes, Strigiformes, Switzerland, herd immunity, house sparrow, blackbirds, ectoparasites
INTRODUCTION

Usutu virus (USUV) is a mosquito-borne flavivirus, which was first isolated from *Culex naevii* in South Africa in 1959 (Williams et al. 1964). Within the first documented emergence of USUV outside Africa, it caused fatalities in birds, especially in wild blackbirds (*Turdus merula*) and captive great grey owls (*Strix nebulosa*) in the region of Vienna, Austria, between 2001 and 2005 (Weissenböck et al. 2002, Weissenböck et al. 2003, Chvala et al. 2004). Subsequently the virus spread to Hungary (Bakonyi et al. 2007) and Italy (Manarolla et al. 2010), and caused outbreaks in free and captive wild bird populations. Although closely related to important human pathogens, such as Japanese encephalitis virus and West Nile virus, human disease unequivocally caused by USUV has not been reported previously; although USUV was isolated from a patient with fever and rash in Africa (Digoutte and Adam 2007), and viral RNA was detected in a patient with rash in Austria (Weissenbock et al. 2007). Recent reports, however, described a neuroinvasive USUV infection in a patient with diffuse large B cell lymphoma (Pecorari et al. 2009), and another neuroinvasive case in a patient who received an orthotopic liver transplant (Cavrini et al. 2009). Both cases occurred in Italy, in late summer, 2009.

Pathogenicity of USUV in avian species varies. While domestic chickens (*Gallus domesticus*) (Chvala et al. 2005) and domestic geese (*Anser anser f. domestica*) (Chvala et al. 2006) seem to be resistant to USUV infection, especially blackbirds and owls were severely affected in the Austrian outbreak (Weissenböck et al. 2002, Weissenböck et al. 2003). Pathologic alterations consisted of macroscopic enlarged liver and spleen, and histological of necrotizing hepatitis, myocardial degeneration, and neuronal necrosis (Weissenböck et al. 2003). Complete genome analysis and comparisons of the reference strain of the South Africa USUV and the Austrian isolates exhibited 97% nucleotide and 99% amino acid identity (Bakonyi et al. 2004).
From the end of July through mid September 2006, considerable, acute mortality in wild and captive Passeriformes and Strigiformes was observed around Zurich Zoo, Switzerland. Investigations determined Usutu virus as the causative agent. Since then the virus caused a significant mortality in captive Strigiformes during late summer month every year. In view of the spread of USUV through Central Europe, the aims of the present study were (i) to describe clinical and pathological findings in affected avian species on the basis of the situation in Switzerland, and (ii) to compare pathological alterations, the genomic structure and the disease epidemiology in Switzerland to the Austrian emergence.

MATERIALS AND METHODS

Clinical History

From the end of July through mid-September 2006 a considerable mortality in wild and captive Passeriformes and Strigiformes was observed around Zurich Zoo, Switzerland. All animals were found within a range of 2 km². Blood was collected in six captive Strigiformes from the right jugular vein for haematology and blood chemistry one to two days before death. The samples were analyzed within six hours at a clinical laboratory (Clinical Laboratory, Vetsuisse Faculty, University of Zurich). Cloacal swabs were collected from dead and sick birds to test for avian influenza. Swabs were placed in Viral Culturette™ tubes (Becton Dickinson Microbiology Systems, Sparks, USA) and were analyzed within 24 hours of collection according to described procedures at the Reference Laboratory, Institute for Veterinary Bacteriology, Vetsuisse Faculty, University of Zürich (Dalessi et al. 2007). Blood was assayed for Newcastle Disease Virus antibodies using an haemagglutination-inhibition test (Wunderwald and Hoop 2002).
Due to the results of the pathological and virological analysis and an epidemiological evaluation, all Strigiformes at Zurich Zoo born or acquired after October 2006 were housed indoors from July to September every year. Again from mid August to the end of September 2007, an increased number of dying wild Passeriformes were observed in the vicinity of Zurich Zoo. Found carcasses in moderate to good postmortem condition were sent for necropsy and further virological analysis. In addition, one blackbird which was found dead 15 km outside the zoo grounds was included in the study because the animal was observed showing clinical signs (incoordination) indicating USUV infection before death.

While in 2008 no suspicious USUV-associated avian case was detected at Zurich Zoo, in 2009, between July and September, captive Strigiformes and wild Passeriformes died either acutely or were seen with neurological disturbances at Zurich Zoo. After the recognition of the increased mortality in Strigiformes remaining animals were treated for ectoparasites and transferred to indoor housing until end of September.

Climatic factors were measured at the weather station Zürich Fluntern, of the Swiss Weather Service and included: monthly mean air temperature in centigrade and monthly sum of rainfall from January 2000 until December 2009. Climatic factors during the last ten years were compared using a one-way analysis of variance (ANOVA). It was assumed that data from each sample were normally distributed and that each sample had the same variance, the latter were tested by Kolmogorov-Smirnov test and Hartley F$_{\text{max}}$-Test, respectively.

**Pathology**

Necropsies were performed either at the Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, Bern, Switzerland or the Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland between 6 h to 48 h after the animal’s death.
Tissue samples were fixed in 4% buffered formalin for histopathology or immunohistochemistry and, in addition, frozen tissue samples (brain and parenchymal organs) were stored at -40°C for further investigations. After embedding in paraffin wax, 4-μm sections were stained with hematoxylin and eosin. Besides all affected wild birds and captive Strigiformes, all birds were involved in the histopathological study that died within the collection of Zurich Zoo during July and September of each year.

Immunohistochemistry (IHC) was performed on 4-μm paraffin wax section as described previously (Chvala et al. 2004). The avidin-biotin complex (ABC) technique (Weissenböck et al. 1998) and a mouse West-Nile virus (WNV) antiserum were used to identify flavivirus-infected birds. Positive controls consisted of brain sections of WNV-infected and USUV-infected birds, in which the diagnosis had been confirmed by in-situ hybridization (ISH) and sequencing of a reverse transcription-polymerase chain reaction (RT-PCR) amplification product.

In-situ hybridization was used to identify the presence and distribution of USUV-specific nucleotide sequences according the previously described technique (Chvala et al. 2004). Positive controls consisted of brain sections of USUV-infected birds, in which the diagnosis had been confirmed by sequencing of an RT-PCR amplification product. Negative controls consisted of brain sections of WNV-infected birds.

**Reverse transcription polymerase chain reaction and genomic analysis**

Bird organ samples (mainly brain, but occasionally also spleen, liver, kidney, gut, heart or lung) were homogenized in ceramic mortars with sterile quartz sand and suspended (approx. 50 w/v%) in DEPC-treated distilled water. The suspensions were centrifuged at 4,300 ×g for 10 minutes and viral RNA was extracted from 140 μl of the supernatants using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCRs were
performed on the sample RNAs using the QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Each 25 μl reaction mixture contained 5 μl of 5 × buffer (final MgCl₂ concentration 2.5 mM), 0.4 mM of dNTP, 10 U RNasin™ RNase Inhibitor (Promega, Madison, WI, USA), 0.8 μM of the appropriate forward and reverse primers, 1 μl of enzyme mix (containing reverse transcriptase and DNA polymerase) and 2.5 μl of template RNA. The oligonucleotide primer pairs Usu 1155f-1600r, Usu 1537f-2505r, Usu 2328f-3145r, Usu 9170f-9704r, WNV 9031f-10091r, and WNV 10090f-10807r (Bakonyi et al. 2004) were used for the detection of WNV RNA in the sample materials.

Following reverse transcription (30 min at 50°C and 15 min at 95°C), reaction mixtures were subjected to 40 repetitive cycles of polymerase chain reaction (40 s at 94°C, 50 s at 57°C, and 1 min at 72°C). Reactions were completed by incubation at 72°C for 10 minutes, and 10 μl of the samples were electrophoresed in 1.2% agarose gel. Ethidium-bromide stained gels were visualized under UV light.

The nucleotide sequences of selected amplification products were determined by fluorescence-based direct sequencing method; the sequences were aligned to the complete genome sequences of the USUV strains SAAR-1776, Vienna-2001-blackbird, and Budapest-2005-blackbird (Bakonyi et al. 2004). Sequences of the reference strains are deposited in the GenBank database under accession numbers AY453411, AY453412 and EF206350.

RESULTS

Clinical History

Between July 25th and September 20th, 2006 a total of 113 birds (24 captive and 89 wild birds) died acutely at Zurich Zoo, Switzerland and post mortems including further examinations were
performed. Most animals were found dead (n = 94) or were euthanized (19) in extremis during this time. Most affect species were from the order of Passeriformes (91) and Strigiformes (10) (Table 1). The observed clinical signs in wild Passeriformes involved depression, ruffled plumage, incoordination, seizures, and peracute death. Hematology and blood chemistry values were within reference ranges (ISIS 2002) in five Strigiformes. One pygmy owl (Glaucidium passerinum passerinum) had a mild leucocytosis (??) compared to reference values (ISIS 2002).

All animals were found in a range of 2km². Though information of state veterinarians and several press announcements were released, no other abnormal die off of wild or captive Passeriformes or Strigiformes in Switzerland was observed.

The following year, from mid August to the end of September, a total of 44 wild Passeriformes were found dead on Zoo grounds. In addition four captive Strigiformes were euthanized due to injuries and severe dyspnea during this time. Three Strigiformes and found carcasses of wild birds, in moderate to good post-mortem condition (20), were suspicious for USUV infection at necropsy (Table 1). Neurological signs in wild or captive birds at Zurich Zoo were not observed in autumn 2007. But one blackbird was observed with incoordination 15 km outside the zoo grounds. The bird died during transport by a private person to the veterinary clinic.

While in 2008 neither Zurich Zoo nor the University of Zurich found a suspicious case of USUV infection in any captive or wild avian species, in August 2009 three acute deaths suspicious for USUV infection occurred in captive Strigiformes at Zurich Zoo. As a consequence, all remaining captive Strigiformes were again treated for ectoparasites and transferred to indoor housing units. No obvious die off in wild avian species was observed.

Climatic conditions were not significant different between seasons and years over the last ten years (p >.0.05), although a slight increase in average temperature and decrease in rainfall was observed (Figure 1).
Pathology

In total, 149 animals were examined histopathologically, consisting of birds from the orders of Passeriformes (74), Strigiformes (21), Anseriformes (27), Ciconiiformes (6), Galliformes (11), Piciformes (1), Psittacidae (1), Coraciformes (2), Trogoniformes (1) and Phoenicopteriformes (5). Examined birds were in acceptable to good post-mortem condition. Forty nine percent (73) of investigated animals had typical pathological lesions for USUV infections described previously (Weissenböck et al. 2002). The nutritional status was generally moderate to poor in wild birds, and poor to good in captive animals. Necropsy revealed a marked splenomegaly, a slightly swollen liver, and hyperemic lungs in most animals (Figure 2). While all affected wild birds (61) were within the order of Passeriformes, confirmed USUV infections in captive birds (12) were found exclusively in the order of Strigiformes. None of the other orders showed typical lesions indicative for USUV infection.

Histopathologic lesions were very discrete and consisted mainly of neuronal necrosis, leucoeytolysis in and around the brain blood vessels, and miliary necroses in the liver. Additionally, scattered cellular necrosis associated with minimal inflammatory reaction was frequently observed in heart muscle tissue, and rarely in kidneys and spleen (Figure 3).

No avian influenza or Newcastle disease virus was found in any bird.

Reverse transcription polymerase chain reaction and genomic analyzes

The diagnosis ‘USUV infection’ was confirmed by immunohistochemistry (IHC) and reverse transcription polymerase chain reaction (RT-PCR). RT-PCR with WNV specific primers and ISH with a WNV-specific probe were negative in all analyzed samples. All USUV positive animals were within the order of Passeriformes and Strigiformes.
The nucleotide (nt.) sequences of selected amplification products were determined and compared to the Austrian (blackbird 2001), Hungarian (blackbird 2005) and South-African (SAAR 1776) strains’ sequences. Within the E protein coding region (between nt. positions 1175 and 2490) the nucleotide sequence of the Zurich 2006 virus differed in one nucleotide from the other three investigated sequences. (At nt. position 1524 of the complete USUV genomes a T is found, which was substituted to C in the Zurich strain.) In the NS5 protein coding region (between nt. positions 9170-9704) two substitutions were identified: at nt. position 9549 the Austrian and Hungarian strains contain T, while the South-African and Swiss (2006) viruses have C in this locus. In position 9669 the Zurich (2006) viruses nucleotide T differed from the other three sequences (C). The third investigated genome section was the partial NS5 and 3’ untranslated region (3’UTR), between nt. positions 10128 and 10736. In position 10289 the Swiss viruses from 2006 and 2009 contain T, while the other three viruses have C. In position 10311 the Vienna 2001 contains A, the other investigated viruses contain G. In position 10374 the Zurich 2009 virus contains C, while the others (including Zurich 2006) have T. All these substitutions are transitions and none of them changed the putative amino acid sequence of the precursor polypeptide (Table 2).

**DISCUSSION**

The current study documents the spread and maintenance of USUV in Central Europe. It appears that this mosquito-borne flavivirus, which had never been observed outside tropical and subtropical Africa until the Austrian outbreak in 2001 (Weissenböck et al. 2003), is affecting the native avian populations of Central Europe. Comparisons of pathologic alterations revealed similar lesions between the Austrian, Hungarian, Italian and the Swiss USUV emergence. Supported by partial nucleotide sequence analysis with >99% identity between the viruses which
emerged in Zurich in 2006, in Vienna in 2001, in Budapest in 2005, and in Milan 2006 it is suspected that a particular strain of USUV is spreading in central Europe. The major clinical and pathological findings were comparable in all Central European locations. Clinical symptoms are rarely described in previous studies due to the fast disease process. Affected birds with confirmed USUV infection showed similar unspecific clinical signs with neurological disturbances suspicious for an encephalitis shortly before death. Nevertheless, animals must have suffered from the disease for several days, because most animals were in a compromised nutritional status when found at a time when food resources for wild Passeriformes seem unlimited in affected locations. Therefore, neurological sings and / or sudden death with pathological findings such as hepato- and splenomegaly, neuronal necrosis, and necrotic changes in liver and heart are indicative to consider USUV infection. Often, however, not all of the lesions mentioned above are present and diagnosis requires confirmation by the demonstration of USUV, either by conventional or molecular virological methods or by the detection of viral signals within tissue sections. In particular, ISH with a USUV-specific probe is an excellent confirmatory tool as it has been already proven in previous studies (Chvala et al. 2004).

With regard to lesions and viral distribution in tissues, the findings largely corresponded to those in birds in the previous USUV outbreaks (Bakonyi et al. 2004, Chvala et al. 2004). Minor differences were observed in affected species. While in the Austrian outbreak more blackbirds were dying of USUV infection, house sparrows were the most affected species in the Swiss emergence. However, in general it seems that USUV infections are a great concern in Passeriformes and Strigiformes while previous studies have shown that USUV is of limited pathogenity in the domestic chicken (*Gallus domesticus*) and geese (*Anser anser f. domestica*) (Chvala et al. 2005, Chvala et al. 2006). If captive, exotic Passeriformes are as sensible to USUV
as the domestic house sparrow is unknown, especially since most of these exotic birds were housed indoors with less contact to the transmitting vectors.

Current USUV outbreaks seem to be strongly limited on selective locations in Austria, Hungary, Italy, and Switzerland (Dorrestein et al. 2007, Manarolla et al. 2009). Furthermore, a serological study suggests exposure of wild, nonmigratory birds in the United Kingdom to this virus as well (Buckley et al. 2003, Buckley et al. 2006). Recently specific RNA sequences of another USUV strain were detected in a pool of Culex pipiens mosquitoes, which were collected in Spain; however, USUV-associated avian mortality was not reported in the country (Busquets et al. 2008). It appears that zoological facilities have an important role in the detection of USUV infection. Similarly to the Austrian and Italian USUV outbreak (Weissenböck et al. 2002), the emergence of USUV in Switzerland was first recognized in a zoological facility. Zoological facilities with their intensive health surveillance program and personnel trained to observe animals for clinical signs of disease have proven to be of major importance in the early detection of emerging diseases, including spongiform encephalopathies and West Nile virus (Kirkwood and Cunningham 1994, Steele et al. 2000). Vulnerable collections, a wide variety of species from different habitats, and the preventative health management can be an exceptional combination for animal health surveillance as well as for early detection of emerging diseases and zoonoses. In addition, avian carcasses seem to disappear quickly in the wild. In the Austrian outbreak most birds were mainly found in urban areas in Vienna, in gardens or parks, rather than in the natural environment. Similar in Italy, USUV infections were only diagnosed within the collection of a private owl breeder (Manarolla et al. 2009). In Switzerland, almost all affected birds were found within Zurich zoo. It seems reasonable that USUV is also present outside these well defined grounds, but local wildlife, e.g. red fox (Vulpes vulpes), and domestic predators, e.g. cat (Felis
catus), will probably use the affected dead birds as an easy food source and carcasses remain undetected.

Various theories are discussed for the emergence of USUV in the different locations in Europe. Whether a virulent USUV strain was introduced either via importation of infected birds, by migratory birds, or increased international travel activities and thus by importation of infective vectors, remains hypothetic. It seems rather unlikely that the virus was introduced by migratory birds every year. Comparison of the sequences of the European and the South African USUV strains revealed that with 95% amino acid identity there was sufficient differences in specific amino acid substitutions to conclude that these viruses have evolved independently (Weissenböck et al. 2002), while the Central European strains seem to be closely related to each other and share 99% amino acid identity. It appears reasonable to assume that the USUV has been introduced to the region of Vienna from Africa once, and has established an efficient transmission cycle between local bird and mosquito species since then (Meister et al. 2008). In recent years the USUV is now slowly radiating to neighbouring federal states and countries. It seems that the strains present in Switzerland, Italy, Hungary, and Austria, however, are highly pathogenic and affected areas are extending after some years of rest. The current climatic conditions in Europe, with unusual mild winters, might be of special epidemiologic interest if the virus might accelerate its spread though Europe causing higher avian mortalities in the future. How the virus strain is spreading through Europe or even has been introduced to Europe is, however, still unknown. Remarkably, since USUV appeared in Europe the virus was always found around larger western cities with major international trade activities and important airports. If the introduction of this exotic virus to the different locations was facilitated by human travel activity or by animal trade, or whether disease surveillance in these areas is simply superior (as discussed above), needs to be determined.
In current European locations with USUV activity, avian mortality decreased within a year. In addition, it seems that the preventative health plan for the captive Strigiformes protected the remaining collection and additions from acute disease and reduced mortalities until 2009, when the Strigiforme collection was again housed outdoors during late summer months as before in 2006. It seems important to find out how a safe USUV immunity can be established in a limited number of captive Strigiformes - especially since mortalities in free-ranging Passeriformes were reduced significantly over the years since 2006. The reason remains currently unknown. However, experience from the well documented Austrian USUV outbreak (Weissenböck et al. 2002, Weissenböck et al. 2003, Chvala et al. 2004, Chvala et al. 2007) indicates that virus and avian host immune system adapt well to each other and a high degree of overall immunity in the affected populations of susceptible host species (“flock immunity”) seems to develop over time. As a result, the infections are clinically inapparent and avian mortality and pathological changes do not usually occur in previously exposed individuals (Endy and Nisalak 2002, Malkinson et al. 2002, Buckley et al. 2003). Therefore, indoor housing seems to prevent captive animals from acute USUV-associated death, but also might prevent the development of flock immunity in threatened species. Experience from the Swiss outbreak suggest to house young of the year and additions indoors during the first two seasons of USUV activity. The following years a regular treatment against ectoparasites might help to reduce virus transmittance below the minimal virus concentration and minimal exposure might induce protective immunity against virulent infections in a range of bird species. A vaccination is currently unavailable and the idea that another flavivirus vaccine, like the West-Nile-Virus vaccine, induces a cross protection against USUV seems rather unlikely, especially since the established equine WNV vaccine resulted in variable protection in various bird species (Johnson 2005, Okeson et al. 2007).
CONCLUSION

The present data demonstrate that the area of proven USUV activity in Austria has extended its range in Central Europe. The virus was introduced to Switzerland and Italy presumably in 2006 and has established a transmission cycle there between local bird and mosquito species. This means that further episodes of increased avian mortality in the forthcoming years must be expected in these areas, with impact on wild and captive bird populations, predominantly Passeriformes and Strigiformes. Furthermore, the possibility of the spread of USUV through whole Europe during the following mosquito seasons must be considered and an increased mortality in Passeriformes and Strigiformes must be expected until protective “flock immunity” is established. Collections of valuable and endangered Passeriformes and Strigiformes should be housed indoors or treated at acceptable intervals against ectoparasites between July and September.

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Figure Legends.

Figure 1: Diagram of climatic conditions at the weather station Fluntern, Zürich, Switzerland, over the last 10 years.
Figure 2: Postmortem appearance of a blackbird (*Turdus merula*) with a confirmed USUTU virus infection. The spleen (S) is severely swollen.
Figure 3: Histopathology of a blackbird (*Turdus merula*) brain, in the cerebral cortex region, showing focal neuronal necrosis (N), accompanied by endothelial swelling and vascular (capillary) necrosis (E). H&E x100. Bar = ??
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Table 2: Nucleotide sequences of selected amplification products of the Swiss USUV outbreak were determined and compared to the Austrian (blackbird 2001), Hungarian (blackbird 2005) and South-African (SAAR 1776) strains’ sequences. Changes of the nucleotide sequence of the Zurich 2006 virus differed in several regions to the other three investigated sequences.
Table 1: Numbers of wild and captive avian species died at Zurich Zoo in the period from July to September in the years from 2006 to 2009.

<table>
<thead>
<tr>
<th>Order</th>
<th>Common name</th>
<th>Scientific name</th>
<th>Housing</th>
<th>Nr of death in the year of 06/07/08/09</th>
<th>Pathology indicative for USUV</th>
<th>USUV PCR positive animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passeriformes</td>
<td>House sparrow</td>
<td>Passer domesticus</td>
<td>Wild</td>
<td>50 / 24 / 0 / 0</td>
<td>33 (33)³</td>
<td>30 (42)²</td>
</tr>
<tr>
<td></td>
<td>Black bird</td>
<td>Turdus merula</td>
<td>Wild</td>
<td>34 / 14 / 0 / 0</td>
<td>24 (26)</td>
<td>20 (26)</td>
</tr>
<tr>
<td></td>
<td>Blue tit</td>
<td>Parus caeruleus</td>
<td>Wild</td>
<td>1 / 2 / 0 / 0</td>
<td>2 (3)</td>
<td>1 (2)</td>
</tr>
<tr>
<td></td>
<td>European greenfinch</td>
<td>Carduelis chloris</td>
<td>Wild</td>
<td>1 / 0 / 0 / 0</td>
<td>1 (1)</td>
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<td>European robin</td>
<td>Erithacus rubecula</td>
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<td></td>
<td>Barn swallow</td>
<td>Hirundo rustica</td>
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<td>Ultramarine grosbeak</td>
<td>Cyanocompsa brissonii</td>
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<td>Bali myna</td>
<td>Leucopsar rothschildi</td>
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<td>Purple glossy starling</td>
<td>Lamprotornis purpureus</td>
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<td>Blue-necked tanager</td>
<td>Tangara cyanicollis</td>
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<td>Strigiformes</td>
<td>Tengmaml’s owl</td>
<td>Aegolius funereus</td>
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<td>5 / 1 / 0 / 0</td>
<td>4 (6)</td>
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<tr>
<td></td>
<td>Great grey owl</td>
<td>Strix nebulosa lapponica</td>
<td>Captive</td>
<td>3 / 2 / 0 / 3</td>
<td>5 (8)</td>
<td>5 (6)</td>
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<tr>
<td></td>
<td>Hawk owl</td>
<td>Surnia ulula ulula</td>
<td>Captive</td>
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<td>2 (3)</td>
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<td></td>
<td>Snowy owl</td>
<td>Nyctea scandiaca</td>
<td>Captive</td>
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<tr>
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<td>Pygmy owl</td>
<td>Glaucidium passerinum</td>
<td>Captive</td>
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<tr>
<td>Anseriformes</td>
<td>Upland goose</td>
<td>Chloephaga picta</td>
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<td></td>
<td>Falkland steamer duck</td>
<td>Trachyeres brachypterus</td>
<td>Captive</td>
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<td>0 (2)</td>
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<tr>
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<td>Common eider</td>
<td>Somateria mollissima</td>
<td>Captive</td>
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<td>African pygmy goose</td>
<td>Nettapus auritus</td>
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<td>0 / 3 / 1 / 1</td>
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<td>White faced whistling duck</td>
<td>Dendrocygna viduata</td>
<td>Captive</td>
<td>1 / 0 / 0 / 1</td>
<td>0 (2)</td>
<td>0 (1)</td>
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<tr>
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<td>Lesser white fronted goose</td>
<td>Anser erythropus</td>
<td>Captive</td>
<td>1 / 0 / 1 / 0</td>
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<td>European golden-eye</td>
<td>Bucephala clangula clangula</td>
<td>Captive</td>
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<td>Falcated teal</td>
<td>Anas falcata</td>
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<td>Baikal teal</td>
<td>Anas formosa</td>
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<td>Sarkidiornis melanotos carunculatus</td>
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<td>Mandarin duck</td>
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<td>Bar-headed goose</td>
<td>Anser indicus</td>
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<td>Population</td>
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<tr>
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<td>Crested wood partridge, <em>Rollulus roulor</em></td>
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<td>Psittacidae</td>
<td>Patagonian conure, <em>Cyanoliseus patagonus</em></td>
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<td>0 (1)</td>
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<tr>
<td>Trogoniformes</td>
<td>White tailed trogon, <em>Trogon viridis</em></td>
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<td>Phoenicopteriformes</td>
<td>Chile flamingo, <em>Phoenicopterus chilensis</em></td>
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*Analyzed samples*