

Usutu Virus Epizootic and *Plasmodium* Coinfection in Eurasian Blackbirds (*Turdus merula*) in Flanders, Belgium

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ABSTRACT: At the end of the summer of 2016, unusually high levels of mortality were detected in Passeriformes and Strigiformes in Flanders, Belgium, mainly in European Blackbirds (*Turdus merula*). A passive surveillance program demonstrated a widespread Usutu virus outbreak and revealed a coinfection with *Plasmodium* in 99% of the dead passerine birds that were necropsied.

Usutu virus (USUV), a mosquito-borne flavivirus originating from Africa, has previously caused substantial mortality in European birds (Cadar et al. 2017). Passeriformes, especially Eurasian Blackbirds (*Turdus merula*) and House Sparrows (*Passer domesticus*), as well as Strigiformes such as the Great Gray Owl (*Strix nebulosa*), are highly susceptible. However, mortality in other bird species (Steinmetz et al. 2011; Van Borm et al. 2017) has been recorded in Europe. Infected birds can exhibit nonspecific and neurological symptoms before death (Steinmetz et al. 2011). Multiple introductions, with subsequent adaptation and spread to native vectors and host populations, resulted in distinct European lineages causing epizootics across various geographic European locations (Engel et al. 2016; Cadar et al. 2017). Herd immunity has been observed, suggesting that USUV could evolve toward an endemic status in Europe (Meister et al. 2008; Engel et al. 2016).

The first case of USUV in Belgium was reported in a Bullfinch (*Pyrrhula pyrrhula*) and a Great Spotted Woodpecker (*Dendrocopos major*) in 2013 (Garigliany et al. 2014). In 2016, an important increase of morbidity and mortality was observed in different bird species in the provinces of Antwerp, Limburg,

and Flemish Brabant, coinciding with similar events in neighboring France, Germany, and the Netherlands (Cadar et al. 2017), and in the southern part of Belgium (Garigliany et al. 2017).

Usutu virus detection in six Eurasian Blackbirds found at the end of August 2016, initiated a first passive surveillance program until October 2016 in Flanders. Wildlife rescue centers (WRC) of Opglabbeek (Limburg), Herenthout (Antwerp), and Ostend (West-Flanders) were requested by the Belgium Agency for Nature and Forests (ANB) to freeze and submit deceased susceptible bird species. In total, 108 birds, comprising 99 Eurasian Blackbirds, two Tawny Owls (*Strix aluco*), two Song Thrushes (*Turdus philomelos*), and five Mistle Thrushes (*Turdus viscivorus*), were collected by the WRCs of Opglabbeek and Herenthout. No birds were recovered from Ostend.

To determine USUV dispersion (Fig. 1), nucleic acids were extracted from supernatant of homogenized pooled brain tissue (High Pure Viral Nucleic Acid Kit, Roche Diagnostics, Vilvoorde, Belgium). Forty-three pools (\leq three birds/pool) (pooling based on species, localization, and timing) were analyzed by an adjusted in-house NS2a-West Nile virus (WNV) real-time reverse-transcription-PCR protocol (AgPath-ID™ One-Step RT-PCR Kit, Life Technologies, Carlsbad, California, USA), allowing specific USUV detection. Primers (USUTU-NS2a-fw: 5'-GTYAGTGCCYAYCGGAGTGAC-3'; USUTU-NS2a-rev: 5'-CCCACAATAGCCGGAACAGTCAATC-3') were used at final concentrations of 0.9 μ M (Eurogentec, Liège,

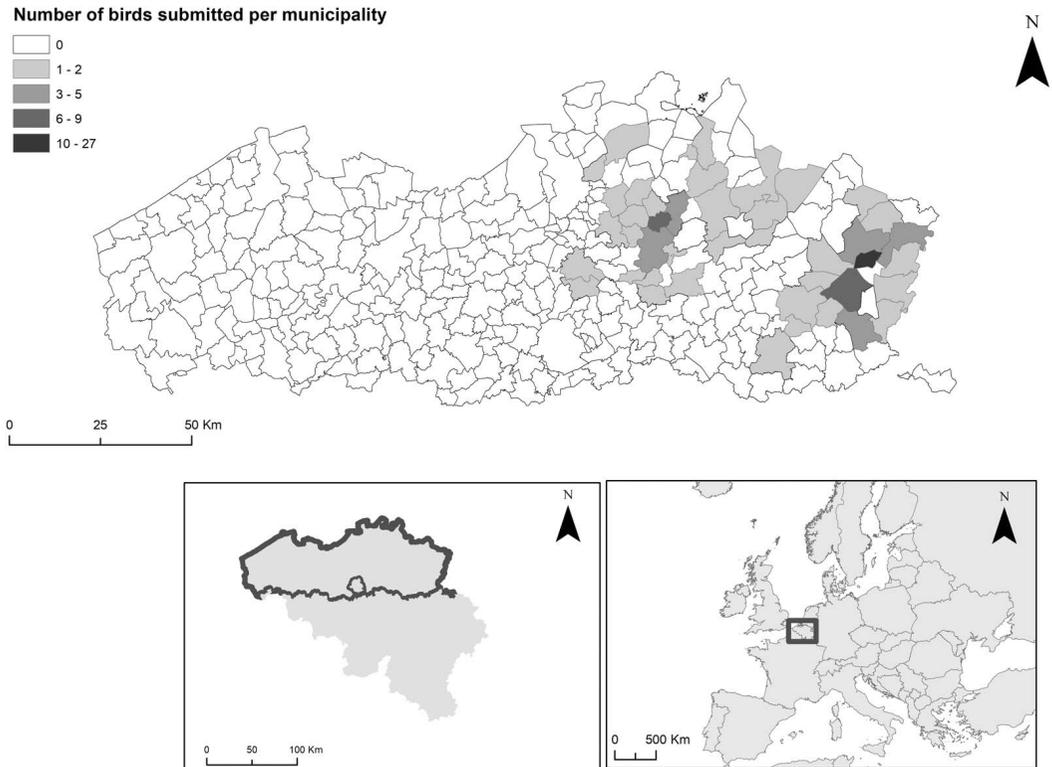


FIGURE 1. The geographical spread of Usutu virus in Flanders (Belgium) is depicted by the number of birds submitted per municipality, encoded by different shades of grey on the map. In total, 44 municipalities submitted 108 birds for the Usutu virus passive surveillance program in 2016 (tested by pooling). All the pools tested positive for Usutu virus.

Belgium), together with a 0.4 μM final concentration of WNV FamTamra-probe (Dridi et al. 2013). All pooled samples tested USUV-positive (Crossing point-values <40).

The remaining carcasses were placed in -70 C until necropsy and further pathogen investigation. Of the 108 birds, 87 Eurasian Blackbirds, one Song Thrush, and three Mistle Thrushes were necropsied. The most remarkable macroscopic findings on necropsy were splenomegaly (80%, 73/91), hepatomegaly (70%, 64/91), and crusty cloacal skin lesions (73%, 66/91). The presence of nematodes, cestodes, and acanthocephalids were considered incidental findings, although some birds exhibited heavy infections. Granuloma within the lungs or air sacs were observed in six Blackbirds; however, no underlying cause was identified. Adult ticks or nymphs were recovered from the head and/or cervical

region of 15 Eurasian Blackbirds and one Mistle Thrush. Subcutaneous edema of the head or cervical region was observed in five Blackbirds, four of which also possessed ticks; however, no subcutaneous edema was present around the tick attachment site.

Apart from USUV, avian malaria, caused by *Plasmodium* and *Haemoproteus* and endemic in Europe (Bentz et al. 2006), has occasionally caused mortality in European birds, displaying similar clinical and pathological symptoms as USUV (Dinhopl et al. 2015). Moreover, coinfection of *Plasmodium* and USUV has been suggested to increase the likelihood of mortality in infected birds (Rijks et al. 2016). Cytology of the lungs, liver, spleen, and kidney for three of the six initial Blackbirds revealed intracellular merozoites within erythrocytes, polychromatophils, and macrophages, a mixed inflammatory infection in various

organs, and in one case, schizonts within the lung tissue. Subsequently all the Passeriformes were additionally tested for avian malaria. The cytochrome-B PCR (Bensch et al. 2000) was performed on DNA extracted from liver tissue (Isolate II Genomic DNA Kit, Bioline, Luckenwalde, Germany). *Plasmodium relictum* (100% query cover and identity to KM361491.2) served as positive control. A total of 99% (90/91) of the necropsied birds tested positive for *Plasmodium* or *Haemoproteus*. The PCR-products of the initial six Eurasian Blackbirds were sequenced (Eurofins, Genomics GmbH, Ebersberg, Germany) and compared to the NCBI database using the BLAST search tool, revealing the presence of *Plasmodium* JF411406.1 (100% query cover and identity) in three Blackbirds, and *Plasmodium* KJ488698.1 (query cover 98%, identity 100%) in one Blackbird. Two birds, displaying double peaks on their chromatograms, suggestive for mixed infections (Dinhopl et al. 2015), were infected with *Plasmodium* spp.

The long, warm summer of 2016, conducive to a high vector-abundance, likely mediated the USUV outbreaks in Belgium and neighboring countries (Cadard et al. 2017). The outbreak seemed regionally confined to the northeast of Belgium (Limburg, Antwerp, and Flemish Brabant) in this study and an additional report about positive cases in the southeast of Belgium (Garigliany et al. 2017).

A high prevalence of *Plasmodium* in Eurasian Blackbirds has been demonstrated before (Bentz et al. 2006), and was confirmed here. Nevertheless, due to lack of historic avian population data, it is not known if *Plasmodium* was highly prevalent before the outbreak in 2016, if its virulence increased, or if avian malaria coemerged and possibly interacted with USUV. The discrepancy between the cytology and PCR results in the initial six Eurasian Blackbirds tested is intrinsically linked to differences in sensitivity between visual and biomolecular detection and is related to the infection intensity (Bentz et al. 2006).

The impact of concurrent vector-borne infections, such as USUV and avian malaria

(Rijks et al. 2016), on disease outcome deserves further attention. Follow-up of the situation in Flanders during the forthcoming years, by screening vectors for the presence of USUV in combination with viro- and serosurveillance of birds, is warranted to better understand epidemiological features, long term bird population impacts, and to assess whether herd immunity develops.

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