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A study of the circulation of West Nile virus, Sindbis virus, Batai virus and Usutu virus in mosquitoes in a potential high-risk area for arbovirus circulation in the Netherlands, "De Oostvaardersplassen".

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Abstract

The Dutch national nature reserve "De Oostvaardersplassen" is considered a potential high-risk area for arbovirus circulation in the Netherlands: over 6000 hectares of wetland accommodating a diverse and abundant mosquito population, and a wide variety of wildlife. This wildlife includes migratory birds arriving from arbovirus endemic areas in Africa and Central Europe. Here we have continued a combined mosquito and virological survey in this area as a first step to assess the risks for public and veterinary health of this nature reserve as ecosystem for enzootic arbovirus circulation. A combination of three types of traps was used and collected mosquitoes were analysed for the presence of West Nile virus, Usutu virus, Batai virus and Sindbis virus RNA. Sixteen different species, covering six genera were collected, in a total number of 1557 mosquitoes collected in 31 trap nights. Thirteen species are reported field or laboratory vectors for a wide variety of infectious pathogens, including West Nile virus, Usutu virus, Tahyna virus, Rift valley fever virus, Sindbis virus, Batai virus, Lednice virus, *Francisella tularensis* and *Dirofilaria immitis*. No evidence for the circulation of arboviruses was found in the collected mosquitoes.

Keywords: arbovirus, Culicidae, ecology, birds, zoonoses.

Introduction

Emerging infectious diseases caused by arthropod-borne viruses (arboviruses) are of increasing concern worldwide. More than 550 arboviruses are recognized of which nearly 100 are considered as human pathogens (Karabatsos, 1985; Gratz, 2006). In principal, all arboviruses pathogenic to humans are zoonotic, characterized by enzootic circulation among wild animal reservoirs and causing human disease upon spillover transmission to humans that are dead-end hosts. However, arboviruses like denguevirus and chikungunya virus have lost the requirement for amplification in animal hosts and are readily transmitted by mosquito vectors between humans, causing extensive epidemics in the tropics and recently even autochthonous cases in Europe (Rezza *et al*., 2007; Weaver & Reisen, 2010) (Gould *et al*., 2010; La Ruche *et al*., 2010).

A number of human mosquito-borne viruses are maintained in an enzootic cycle with birds as amplifying reservoir hosts and ornithophilic mosquitoes as transmitting vectors. West Nile virus (WNV, flavivirus), Sindbis virus (SINV, alphavirus) and Usutu virus (USUV, flavivirus) are maintained in such avian-mosquito cycle. Their vertebrate reservoirs are largely passeriform birds, with migratory members being responsible for a wide geographic distribution (Hubálek, 2008; Weissenbock *et al*., 2009; Sammels *et al*., 1999; Norder *et al*., 1996; Gratz, 2006; Parreira *et al*., 2007; Kurkela *et al*., 2008; Rappole *et al*., 2000; Rappole & Hubálek, 2003; Koopmans *et al*., 2008; Jost *et al*., 2010). For an efficient local transmission of these viruses, the indigenous bird population should sustain the virus at sufficient viremic levels and local

competent mosquito species are needed to sustain and transmit the virus among the resident birds. Ornithophilic mosquito species will be the most relevant species for establishment of these three viruses in an enzootic cycle (Rappole *et al*., 2000; Higgs *et al*., 2004; Calzolari *et al*., 2010; Francy *et al*., 1989). For transmission to humans, vectors are needed that have a more opportunistic feeding behaviour; feeding both on birds and mammals and serving as bridge vectors. In addition to these three arboviruses, bataivirus (BATV, orthobunyavirus) is thought to be associated in sylvatic foci with birds. Although BATV has principally a domestic animal – mosquito cycle it can persistently infect birds and the virus is thought to spread across Europe through migratory birds similar to WNV, USUV and SINV (Hubálek and Halouzka, 1996).

WNV is the aetiological agent of West Nile Fever (WNF), a vector-borne disease endemic in Africa, East Asia, North-, Central- and South America and Southern Europe (Dauphin *et al*., 2004). The majority of human WNV infections are asymptomatic. American studies estimated that approximately 20% of human infections will result in a mild disease characterized by fever, headache, myalgia and fatigue. Less than 1% of all infected persons develop severe neuroinvasive disease, with a case fatality of approximately 10% (Kramer *et al*., 2007). The recent epidemiological picture of human WNV infections in Europe shows that WNV is actively circulating in Central Europe and the Mediterranean and that transmission to humans occurs on a regular basis during the mosquito season. In recent years sporadic cases of human WNV infections have been identified in Hungary, Italy, France, Portugal, Romania, Russia and Spain. In 2008 for the first time outbreaks of human infections were reported simultaneously from three European countries, *viz.* Hungary, Italy and Romania (Calistri *et al*., 2010). In 2010 human cases were reported from 6 European countries. A few cases were registred in Portugal, Italy, Romania and Hungary (Anonymous, 2010a). An outbreak involving >150 cases was reported from northern Greece. In the Volgograd region in southern Russia > 200 cases were registered (Anonymous, 2010b; Papa *et al*., 2010).

The presence of WNV is not restricted to central and southern Europe as seropositive birds have been found in the UK, Poland and Germany (Buckley *et al*., 2006; Hubálek, *et al*., 2008) (Linke *et al*., 2007; Seidowski *et al*., 2010). Phylogenetically WNV is divided into two main lineages. Lineage 1 is the cause of the majority of outbreaks in North-America and Europe. Lineage 2 circulates endemically in Africa and has only recently be identified in Europe, *viz*. in Hungary (2004-2008), Russia (2007) and Austria (2008) (Bakonyi *et al*., 2006; Weissenbock *et al*., 2009). Lineage 2 WNV was isolated from mosquitoes in the 2010 outbreak in Greece (Anonymous, 2010b). In Europe WNV has been detected in eight different mosquito species , covering 5 genera, in Italy, France, Spain, Portugal, Moldavia, Greece, Romania, Slovakia, the Czech Republic, Ukraine and Belarus. Species reported positive in the field are *Culex pipiens s.l., Cx. modestus*, *Cx. univittatus*, *Coquillettidia richiardii*, *Ochlerotatus cantans*, *Aedes rossicus*, *Anopheles maculipennis s.s.* and *An. atroparvus* (Calzolari *et al*., 2010; Tamba *et al*., 2010; Hubálek, *et al*., 2010; Vazquez *et al*., 2010; Hannoun *et al*., 1964; Filipe, 1972; Labuda *et al*., 1974; Hubálek & Halouzka 1996; Fernandes *et al*., 1998; Savage *et al*., 1999; Esteves *et al*., 2005; Almeida *et al*., 2008; Hubálek, 2008). However the actual role of some of these species in WNV epidemiology remains to be determined. The ornithophilic members of the species complex *Cx. pipiens s.l.* are the most common vector species for WNV both in Europe and the USA.

SINV circulates in Eurasia, Africa and Oceania (Taylor *et al*., 1955; Hubálek, 2008) and is the causative agent of Pogosta disease (Finland) also known as Ockelbo disease (Sweden) and Karelian fever (Russia). Pogosta disease is characterized by arthritis, rash, fatigue, fever, headache and myalgia (Kurkela *et al*., 2005). Outbreaks in Europe of Pogosta disease (human Sindbis virus infections) have thus far emerged in Fennoscandia every seven years since the 1st outbreak was noted in 1974 with hundreds to thousands of clinical cases (Hubálek, 2008). However in 2009 this seven-year cycle did not recur (Sane *et al*., 2010). Two distinct SINV lineages are found; a paleoarctic/Ethiopian and an oriental/Australian lineage (Jost *et al*., 2010). *Tetraonidae* and passeriform birds (especially *Turdidae*) have been implied as amplifying reservoir hosts in northern Europe (Lundstrom *et al*., 2001; Brummer-Korvenkontio *et al*., 2002). In Europe SINV has been detected in *Cx. pipiens s.l., Cx. torrentium*, *Cx. modestus, Culiseta morsitans, Ae. cinereus*, *Cq. richiardii*, and *An. maculipennis s.l.* (Francy *et al*., 1989; Hubálek & Halouzka, 1996; Lundstrom, 1999; Jost *et al*., 2010).

USUV is endemic in Africa (Gratz, 2006). In Europe evidence for USUV circulation in birds exists for Austria, Hungary, Italy, Switzerland, England, Poland and the Czech Republic (Weissenböck *et al*., 2008). In 2009 the first European human cases of USUV-related neurological disease occurred in Italy (Cavrini *et al*., 2009; Pecorari *et al*., 2009). In addition, USUV was detected in serum of organ donors tested in a retrospective WNV screening performed in Italy in the same year (Capobianchi *et al.*, 2010). In Europe USUV RNA has been detected in *Cx. pipiens s.l.* , *Cx. hortensis*, *Cx. territans*, *Culiseta annulata*, *Ae. albopictus* [= *St. albopicta*], *Ae. rossicus* and *Ae. vexans* (Busquets, *et al*., 2008; Weissenböck *et al*., 2008; Tamba *et al*., 2010).

BATV is widely present in Europe, Asia and Africa. In Europe evidence for circulation exists for Norway, Sweden, Finland, Slovakia, the Czech Republic, Croatia, Serbia, Bosnia, Montenegro, Italy, Hungary, Romania, Austria, Portugal, Germany and Belarus (Lundstrom, 1999; Jost *et al*., 2011). Vertebrate hosts are pigs, horses and ruminants. Persistent infections have been reported for several bird species. Human BATV infections are associated with influenza-like illness in Europe and febrile illness in Asia and Africa (Hubálek, 2008; Juriková *et al*., 2009) . Principal vectors in Europe are zoophilic species, *viz. An. maculipennis* s.l., *An. claviger*, *Cq. richiardii*, *Oc. punctor* and *Oc. communis* (Francy *et al*., 1989; Hubálek, 2008).

Areas with favourable ecological conditions for the interaction between resident bird reservoirs, migratory bird reservoirs and competent ornithophilic vectors are at risk for enzootic circulation of WNV, USUV, SINV and BATV. The presence of competent bridge vectors and humans will increase the risk for transmission of these viruses to humans. Outbreaks of WNV are often focused in or near wetlands where large numbers of (migratory) birds are bitten by large numbers of mosquitoes (Koopmans *et al*., 2008). In Germany SINV and BATV were found in mosquitoes trapped in wildlife sanctuaries along the Rhine river that are regularly flooded and with a known high abundance of mosquitoes and high occurrence of migratory birds (Jost *et al*., 2010; Jost *et al*., 2011). The Dutch national park "De Oostvaardersplassen" is considered such a high risk ecosystem for introduction and enzootic circulation of WNV, SINV, USUV and BATV in the Netherlands. The 6000 hectares nature development reserve is of international importance as wetland and habitat for migratory birds, including birds visiting from arbovirus endemic areas in Europe and Africa. The area is renown for it's wide variety in wildlife and richness and abundance of mosquito species (Reusken *et al*., 2010). Over 190 different bird species have been observed in the reserve since 1999, including over 45 bird species of which evidence for WNV infection exists from foreign field studies (Koopmans *et al*., 2008). Wildlife consists amongst others of approximately 500 "Heck Cattle", 1100 "Konik horses" and 2300 red deer. De Oostvaardersplassen has diverse landscapes including marshes, lakes, ponds, ditches, trenches, plains, shrubs, and forests, which create a large diversity of potential mosquito breeding sites. The presence of 365.000 inhabitants of two cities less then 3 km away creates the opportunity for introduction of enzootic circulating arboviruses in the urban cycle through peri-domestic birds and subsequent transmission to humans through bridge vectors (Reusken *et al*., 2010).

As research into the mosquito population and arbovirus circulation in the nature reserve is essential for an assessment of the risks of this area for public health, a combined mosquito and WNV survey in De Oostvaardersplassen was initiated in 2009 (Reusken *et al*., 2010). Thirteen different mosquito species, covering five different genera were collected in this pilot study. Eleven species are reported field or laboratory vectors for a variety of infectious pathogens, including WNV, USUV, SINV, BATV and Tahyna virus, Lednice virus, *Francisella tularensis* and *Dirofilaria immitis*. Although seven potential WNV vector species were collected no evidence for WNV circulation in the mosquitoes (n = 314) was found in 2009. The study was continued in 2010, including a broader screening for arboviruses, *viz.* USUV, SINV and BATV besides WNV. Furthermore, the study was extended with two trap types (carbondioxide-baited CDC-light trap, and Reiters'gravid trap) besides the Mosquito Magnets used in 2009, to collect more specimens of a wider range of mosquito species. This allowed for a trap comparison in collected mosquito-fauna and numbers of collected specimens. The observations are described in this paper.

Figure 1. The study area, Oostvaardersveld, where nine traps were placed.

Materials and methods

Survey design

Adult mosquitoes were collected in the 'Oostvaardersveld', an area of approximately 328 ha in the southeastern corner of De Oostvaardersplassen (Figure 1). The Oostvaardersveld consists of open grassland with sparse shrubs, ponds, marshes, canals, and deciduous forest. A group of approximately 100 Konik horses are present, spending most of their time in an open grassland area in the northeastern part of the Oostvaardersveld. In the middle of this open grassland lies a shallow, permanent pond with high numbers of birds (mostly geese and various duck species). This area of open grassland is surrounded by deciduous forest (mostly *Salix* species). In some parts of this forest, regularly floods occur, creating marshes.

Experimental design

The study was conducted in the same period as the study in 2009 to allow for data comparison. Based on the occurance of WNV outbreaks in Europe during the summer (July-August-September), August was chosen. Mosquitoes were collected at the same 9 sites in the Oostvaardersveld as in 2009 (for detailed description of these sites, see (Reusken *et al*., 2010). Mosquitoes were collected on two occasions for two consecutive days; in week 32 (10-11 August 2010) and in week 34 (24-25 August 2009).

Mosquito collections

In concordance to commonly used WNV entomological surveillance methods in the USA (Lukacik *et al*., 2006; Williams & Gingrich, 2007; Ginsberg *et al*., 2010), two other mosquito trap types were added to the carbon dioxide and octenol-baited Mosquito Magnet traps (MM, Liberty Plus type, American Biophysics) that had been used in 2009: 1) the carbon dioxide (dry ice)-baited New Standard Miniature Incandescent Light Trap Model 1012 (LT, John W. Hock Company), and 2) the infusion-baited CDC Gravid Trap Model 1712 (GT, W. Hock Company). The GT was added to increase the likelihood of collecting WNV-infected female mosquitoes (if present at all) (Lukacik *et al*., 2006; Williams & Gingrich, 2007), using one week old hay infusion: the nutrient rich water in the trap attracts gravid *Culex* females that need to lay their eggs (Reiter, 1983).

The three different mosquito traps per site were used contemporaneously and were placed not closer than 3 and not further away than 5 metres from each other. In total the 27 traps ran continuously during the experimental period, with exception of one GT at location 'I', that was placed on the 10th instead of the 9th of August. The traps were placed in the morning of the 9th of August. The nets were retrieved from all traps after 24 hrs (on the morning of the 10th of August), and replaced with empty nets, which were subsequently retrieved 24 hrs later (11th of August). Retrieved nets with (still alive) mosquitoes were placed in a sealed bag, labelled, and placed in an equally labelled cardboard box for protection (one net per box). These labelled boxes were kept in the car for later mosquito species identification. In week 34, the same procedure was followed: traps were switched on, on the 23rd of August, and retrieved subsequently 24 and 48 hrs later (24th and 25th of August). In those cases where, while handling the traps, mosquitoes were biting, these were collected by using a mouth aspirator.

Mosquito species identification was carried out as described in (Reusken *et al*., 2010).

Statistical analysis.

Paired student-T tests were used to analyse catches of host-seeking mosquitoes. Pairs consisted of nightly catches in both the MM and LT. Catches of the GT were discarded in the analysis, because this trap is not attracting host-seeking mosquitoes. Species of which sufficient numbers were collected, were included in further analysis of trapping efficacy for a specific species.

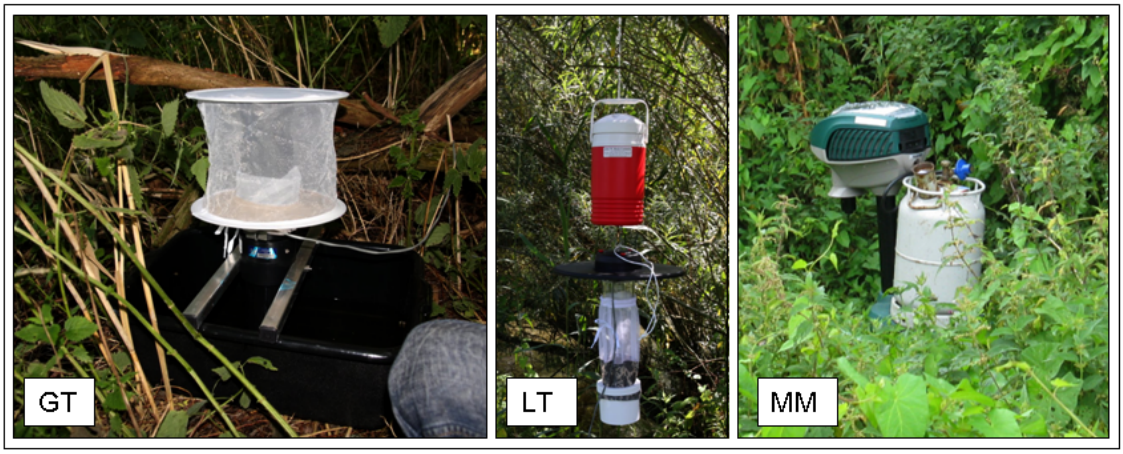

Figure 2. The three types of adult mosquito traps that were used in this study: infusion-baited CDC Gravid trap (GT), dry-ice (carbon dioxide)-baited New standard CDC Light trap (LT) and carbondioxide- and octenol-baited Mosquito Magnet Liberty Plus (MM) trap.

Monitoring for presence of arboviruses

Upon identification, the collected mosquitoes were immediately flash frozen using dry-ice and stored at -80°C until analysis. Mosquitoes were pooled by sampling site, sampling date and species. Mosquitoes were ground using liquid N_2 and pestles, and RNA was extracted using the RNeasy ® RNA Isolation Minikit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and including a Qiashredder step for homogenization and DNaseI treatment. RNA was isolated from pools of no more than five mosquitoes. As a quality control for the homogenization and isolation step, each mosquito pool was processed in the presence of MS2 armored RNA and analysed by RT-PCR as described in (Stevenson, Hymas *et al*., 2008).

The presence of WNV was analysed using a multiplex real-time RT-PCR assay, allowing detection and discrimination of lineages 1 and 2 as described in (Reusken *et al*., 2010). The presence of SINV was analysed by real-time RT-PCR essentially as described in (Jost *et al*., 2010). The mosquitoes were analysed for evidence of USUV circulation by using a real-time RT-PCR assay based on the flavivirus genus wide assay described by (Chao, Davis *et al.,* 2007). Two USUV specific probes were especially designed for this purpose: FAM-ATGAGCCACCACTCAG-BHQ1 and FAM-ATGAGCCACCATTCAG-BHQ1. For the detection of BATV a real-time RT-PCR was designed based on an alignment of BATV S-segment sequences available in GenBank using Kodon 3.6 and Visual OMP ™. Forward primer:ATGATGTCGCTGCTAACACC, reverse primer: CCAGTGGTGTAGAYACGCTTAAAG, probe: Cy5-GCAGTACTTTTGACCCAGAGGTTGCAT-BHQ2.

Results

Entomological survey

Species composition

In total, 1557 mosquitoes were collected. Sixteen different species were found, representing 44% of the Culicidae species diversity of the Netherlands: *Culex pipiens, Cx torrentium, Cx. modestus, Aedes vexans, Ochlerotatus annulipes, Oc. cantans, Oc. detritus, Oc. riparius, Coquillettidia richiardii, Culiseta annulata, Cx. fumipennis, Cx. morsitans, Cs. subochrea, Anopheles claviger, An. maculipennis s.l.* and *An. plumbeus* (Table 1). Fifteen specimens were too damaged for morphological diagnostics up to species level: two *Culex*, 12 *Ochlerotatus*, and one *Culiseta* spp. specimen. In all trap types, males were collected, with a total of 35 specimens (2%). *Culex pipiens*, *Cq. richiardii* and *Cs. annulata* were the most abundant species, with 771, 346, and 235 specimens respectively, together making up for 87% of the collected mosquitoes. Five new species were collected in 2010 in comparison to 2009: *Ae. vexans* (1 specimen), *Oc. annulipes* (31 specimens), *Oc. detritus* (1 specimen), *Oc. riparius* (1 specimen), and *Oc. fumipennis* (41 specimens). On the other hand, two species that were collected in 2009, *Oc. geniculatus* [=*Dahliana geniculata*] and *An. algeriensis*, were absent in the trappings in the subsequent year.

In comparison to 2009 the MM collected a similar amount of mosquitoes in 2010, *viz* 397 vs. 426 specimens. A clear difference in species composition between the collections of 2009 and 2010 is observed, particularly regarding three species: 33x more *Cx. pipiens* s.l., 2x more *Cs. annulata* and 8x less *An. claviger* specimens were collected in 2010 in comparison to 2009 (Table 1).

Trap comparison

Most mosquitoes were collected in the LT (1035 specimens, 67%), followed by the MM (426 specimens, 27%), and the GT (77 specimens, 6%). Manually, a total of 19 mosquitoes were collected, but since these were not collected systematically, they are not included in the trap comparison. With 695 specimens, *Cx. pipiens s.l.* was by far the most abundant species collected with the LT. Only *Cx. pipiens s.l.*, *Cq.*

richiardii and *Cs. annulata* were collected in sufficient numbers for statistical comparison of the three trap types used.

The number of species per trap night that was collected with the LT was significantly higher than the number of species collected with the MM (3.6 ± 0.31 vs. 2.5 ± 0.33 respectively; n = 31; t = 2,77; p = 0,01), see Figure 3.

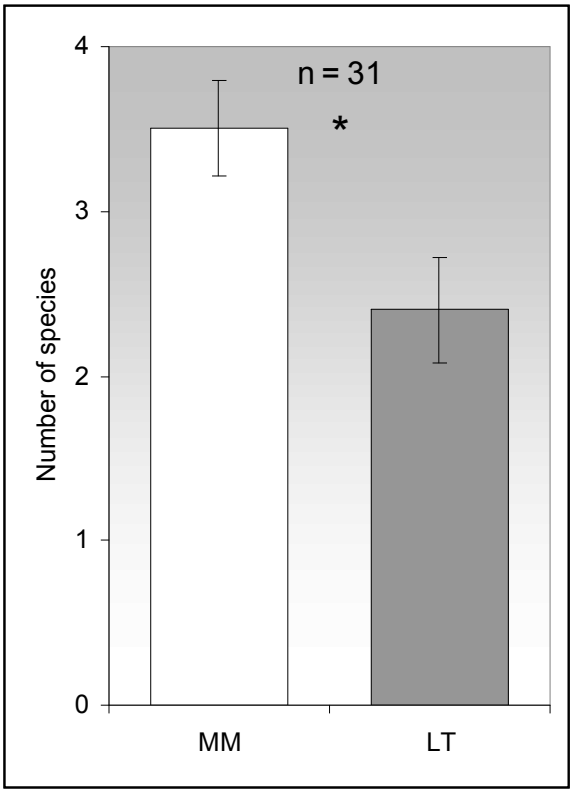

Figure 3. Mean number (+/- SEM) of species collected per trap night (n = 31) with the CDC Light trap (LT) and the Mosquito Magnet Liberty Plus trap (MM).

The number of *Cx. pipiens s.l.* that was collected per trap night with the LT was significantly higher ($p < 0.001$; t = -4.8; n = 32) than the number collected with the MM (21.7 ± 4.4 vs. 0.9 ± 0.8 respectively). For *Cq. richiardii* and *Cs. annulata* no significant differences were found between both trap types.

Table 1. Total number of mosquitoes that were collected per species and trap type: Mosquito Magnet Liberty Plus trap (MM), CDC Light trap (LT), CDC Gravid trap (GT) in 2009 and 2010 in the Oostvaardersveld. Highlighted grey fields indicate WNV vector species.

		2009	2010				
Genus	**Species**	**MM (= Total 2009)**	**MM**	**LT**	**GT**	**Suction tube**	**Total 2010**
Culex	*Cx. pipiens s.l.*	**9**	32	695	44		**771**
	Cx. pipiens/torrentium		12	29	12		**53**
	Cx. torrentium	**2**		10	2		**12**
	Cx. modestus	**1**	1	1			**2**
	Cx. sp.			2			**2**
Aedes/Ochlerotatus	*Ae. vexans*		1				**1**
	Oc. annulipes		16	2	1	12	**31**
	Oc. annulipes/cantans			1			**1**
	Oc. cantans	**7**	2	1		6	**9**
	Oc. detritus			1			**1**
	Oc. geniculatus	**1**					
	Oc. riparius		1				**1**
	Oc./Ae. sp.	**1**	4	8			**12**
Coquillettidia	*Cq. richiardii*	**96**	197	144	4	1	**346**
Culiseta	*Cs. annulata*	**207**	131	93	11		**235**
	Cs. fumipennis		10	29	2		**41**
	Cs. morsitans	**1**		4			**4**
	Cs. subochrea	**1**		1			**1**
	Cs. sp.		1				**1**
Anopheles	*An. algeriensis*	**6**					
	An. claviger	**55**	13	6			**19**
	An. maculipennis s.l.	**6**	4	6	1		**11**
	An. plumbeus	**4**	1	2			**3**
	Total	**397**	**426**	**1035**	**77**	**19**	**1557**

Arbovirus survey

In total 1557 mosquitoes, belonging to six different genera and 16 different mosquito species, were analysed for the presence of WNV lineages 1 and 2, USUV, BATV and SINV (Table 2). None of the analysed specimens were found positive for any of these arboviruses. The WNV-negative mosquitoes collected in the 2009 study (Reusken *et al*., 2010) were analysed for the presence of USUV, BATV and SINV as well. No evidence for the presence of these viruses was found.

Discussion

Mosquito species

A total of 35 different mosquito species are listed as indigenous species for the Netherlands (Verdonschot, 2002). However, a small number of these species are only assumed to be present as they have never actually been collected (Verdonschot *pers. comm*.). In 2009 and 2010 *Oc. atropalpus* was found but it remains unclear whether this species has established (Scholte *et al*., 2009, 2010). In addition, *Aedes aegypti* and *Stegomyia albopicta* were reported in 2010 as being introduced via the import of used tires (Scholte *et al*., 2010). The latter species is repeatedly imported into the Netherlands via the international trade in Lucky bamboo (*Dracaena sanderiana*) since 2005 as well (Scholte *et al*., 2007, 2008). However, these species have not established in the Netherlands. Sixteen different indigenous mosquito species, covering six different genera were collected during the survey. This represents 44% of the total number of species presumed to be present in the Netherlands, and confirms the presence of high mosquito species richness in the Oostvaardersplassen. The (not) trapping of certain species, the species composition and number of collected specimens are influenced by the trap types used, the trapping season, weather conditions, location of the traps and variations between years.

A few words regarding the trap comparison. First of all, it should be noted that whilst the LT and the MM are both designed to collected host-seeking female mosquitoes, GT are designed to attract mosquitoes that are in a different physiological stage. Being gravid they are in need not for hosts, but for suitable breeding sites, therefore responding to different odours than host seeking female mosquitoes. Since the framework of this study is based on estimating disease risk and GT are in general an integrated part of an entomological WNV surveillance, they were included in the set-up, but not included in the trap comparison. Secondly, it should be noted that the traps might have influenced each other, and the total trapping collection. For example, the total amount of odour bait coming from each site with the three traps is higher (more carbon dioxide produced), and the combination of octenol, carbon dioxide, and hay-infusion, is more diverse when compared to only one trap.

Disease vectors

Ten species endemic to the Netherlands are reported as field vectors for WNV in foreign studies of which seven have been collected in this study, *viz. An. maculipennis s.l., Cx. modestus, Cx. pipiens s.l., Oc. cantans, Cs. morsitans, Ae. vexans* and *Cq. richiardii* (Table 1, grey fields). WNV has been isolated from all these species in Europe, with exception of *Ae. vexans* and *Cs. morsitans*. However, these species are observed field vectors in Africa and North America, and North America respectively (Hannoun *et al*., 1964; Filipe, 1972; Labuda *et al*., 1974; Katsarov *et al*., 1980; Fyodorova *et al*., 2006; Koopmans *et al*., 2008; Anonymous, 2011; Berthet *et al*., 1997). In addition, *Anopheles plumbeus* is a reported laboratory vector for WNV (Table 2). 73% of the total number of collected mosquitoes are implicated as WNV vector in literature. *Culex pipiens s.l.* (771 specimens, 67%) and *Cq. richiardii* (346 specimens, 30%) represented the majority thereof. No biotype speciation of the *Cx. pipiens* specimens was carried out, but considering the absence of underground breeding sites, it is likely that the *Cx. pipiens* biotype that is present in the Oostvaardersplassen is the ornithophylic biotype *pipiens* rather than the mammophylic biotype *molestus,* as found in subway systems in the Netherlands (Reusken *et al.*, 2010). The relative abundance of *Cx. pipiens* and *Cq. richiardii* in 2010 could indicate that, upon introduction of WNV in the

area through migratory birds, the virus will be dispersed and maintained in the resident bird population through the large ornithophilic *Cx. pipiens* population while opportunistic *Cq. richiardii* might serve as bridge vector transmitting the virus to horses and humans. Although eight potential WNV vector species were collected in the nature reserve, no evidence for WNV circulation in mosquitoes was found in this study.

SINV vectors. Seven species endemic to the Netherlands are reported vectors for SINV in literature. Six of these have been collected in the Oostvaardersplassen in 2010, *viz. Cx. pipiens s.l.*, *Cx. torrentium*, *Cx. modestus*, *Cs. morsitans*, *Cq. richiardii*, and *An. maculipennis*. These six species represented 73% of the total number of mosquitoes collected in the study. In Scandinavia, where human SINV cases occur, *Cx. pipiens* and/or *Culex torrentium* and *Cs. morsitans* are the probable enzootic vectors. *Ae. cinereus*, an indigenous species for the Netherlands but not found in the Oostvaardersplassen, is considered as bridge vector between humans and birds. In Germany, *An. maculipennis s.l.* were found infected with the virus, but its role as a vector remains unclear (Jost *et al.*, 2010).

BATV vectors. Principal vectors for BATV in Europe are zoophilic species, *viz. An. maculipennis* s.l., *An. claviger*, *Cq. richiardii*, *Oc. punctor* and *Oc. communis* (Hubálek, 2008). In addtion *Ae. vexans* has been implicated as BATV vector in literature. Of these, BATV is mostly found in *An. maculipennis* (Francy, Jaenson *et al.* 1989). Although all these species are indigenous in the Netherlands, the prime BATV vector, *An. maculipennis*, was not collected in large numbers in the study area, suggesting that the area is not specifically suitable for this species. Four species implied in BATV epidemiology, were collected in the nature reserve in 2010, representing 24% of the total mosquito collection.

USUV vectors. USUV appeared for the first time outside Africa in Austria in 2001, causing fatalities in several bird species. In the subsequent years, the virus was detected in the following mosquito species: *Culex hortensis*, *Cx pipiens s.l.*, *Cx. territans*, *Cs.annulata*, *Ae. vexans* and *Ae. rossicus* (Weissenböck *et al.*, 2008). In Spain USUV was isolated from *Cx. pipiens* specimens (Busquets *et al.*, 2008). In Italy USUV was found in *Cx. pipiens* and *Ae. albopictus* [= *St. albopicta*] (Calzolari *et al.*, 2010; Tamba *et al.*, 2010). With the exception of *Cx. hortensis, Ae. albopictus* [= *St. albopicta*] and *Ae. rossicus*, all mentioned species are endemic to the Netherlands. Three of these four species were found in the current study, representing 65% of all collected specimens. It is suggested that *Cx. pipiens s.l.* is the most likely competent vector of USUV in Europe (Weissenböck *et al.*, 2008; Calzolari *et al.*, 2010).

Our observations could mean that although there is arbovirus circulation in the nature reserve, it cannot be detected with the current survey set-up. Enzootic arbovirus transmission may occur only at a low intensity in certain birds and mosquito species. Athough for each of the 4 arboviruses studied here, known vectors were trapped and analysed (specimens of 16 different species), the number of mosquitoes collected in this study is most likely not enough to detect a low endemic virus circulation in the area. This is illustrated in a recent study in Germany: 16,057 mosquitoes in 643 pools were analysed for the presence of SINV and BATV. Ten pools were positive for SINV RNA and only one pool was positive for BATV RNA (Jost *et al.*, 2010; Jost *et al.*, 2011). Furthermore there might be arbovirus circulation in the reserve but not in the area of study. The Oostvaardersveld may not completely reflect the total of the other areas of the nature reserve. Even though the Oostvaardersveld has several waterbodies, these are not comparable in size to the lakes in the other areas. The number of birds visiting the Oostvaardersveld, is relatively low compared to the other areas of the nature reserve. However these areas are not open to the public and researchers. It is necessary to convince policymakers to open the larger areas in De Oostvaardesplassen for a broad and in-depth study into the circulation of arboviruses in the reserve. This study should include analysis of vectors, birds, mammals and employees, inhabitants of the area.

On the other hand the failure to detect WNV, USUV, BATV and SINV in the collected mosquitoes could reflect the absence of circulation of these viruses in The Oostvaardersplassen. Although the viruses might be introduced (repeatedly) in the reserve by migratory birds, the local basic reproduction rate, R0, might be too low for virus establishment in a sylvatic cycle. Further research into factors of influence like the vector competence of the local mosquito population, the susceptibility of the indigenous bird population and ecological factors like temperature, precipitation and occurrence of floods is necessary.

In total 13 of the 16 different mosquito species collected in the Oostvaardersplassen in 2009 and 2010 are reported field or laboratory vectors for infectious pathogens. These include, besides the viruses studied here, Tahyna virus, yellow fever virus, Lednice virus (LEDV), Rift valley fever virus, *Francisella tularensis*, malaria, myxomatosis, and *Dirofilaria immitis* (Table 2). Especially LEDV is a likely other candidate for circulation in the nature reserve. LEDV is also maintained in an avian-mosquito cycle. The vertebrate reservoirs are largely anseriform birds, with migratory members being responsible for wide geographic distribution (Hubálek, 2008; Weissenbockk *et al*., 2009). Natural foci of LEDV infections occur mainly in wetland ecosystems like the Oostvaardersplassen (Hubálek, 2008). Lednice virus circulates in birds in the Czech Republic and Romania. No evidence for disease in mammals, including man, exists sofar (Hubálek, 2008).

Table 2. Overview of diseases putatively transmitted by mosquito species collected in De Oostvaardersplassen.

	Role as vector in diseases																	
Mosquito species	Anaplasmosis	Batai	Canine filariasis	Dirofilaria immitis	Filariasis	Lednice	Lyme	Malaria	Myxomatosis	Rift Valley Fever	Setaria labiatopapillosa	Sindbis	Tahyna	Tularaemia	Usutu	West Nile	Filariasis	Yellow fever
Aedes vexans										2					3			
Anopheles algeriensis								1										
An. claviger	1	1	1				1	2	1		1		1	1				
An. maculipennis s.l.																		
- sensu stricto		1		1				2*	1				1	1		1		
- atroparvus				1				2	2				1	1		2		
- messeae		1	1					2*	1				1	1		1		
An. plumbeus					4			4								4		
Coquillettidia richiardii		2											2			2		
Culex modestus				2		2			2			2	2	2		2		
Cx. pipiens s.l.		2		2,4						2		2	2,4		2	2		
Cx. torrentium												2						
Culiseta annulata									2				1		2			
Cs. morsitans												2*						
Ochlerotatus cantans									2				2			2		
Oc. geniculatus													2			4		4

Taken from (Schaffner *et al.,* 2001) and adapted based on (Poncon *et al.,* 2007, Weissenböck *et al.,* 2008). 1) probable vector
2) field detected and confirmed role as transmitter
3) field detected
4) lab experiment
* minor role

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References

Almeida, A.P., Galao, R.P., Sousa, C.A., Novo, M.T., Parreira, R., Pinto, J., Piedade, J. & Esteves, A. (2008) Potential mosquito vectors of arboviruses in Portugal: species, distribution, abundance and West Nile infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102,** 823-32.

Anonymous (2010a) Review of epidemiological situation on West Nile virus infection in the European Union. ECDC Threat Assessment. September 2010.

Anonymous (2010b) West Nile virus-Eurasia: Russia, Greece. Promedmail Archive Number 20100902.3141.

Anonymous. (2011) http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm#06. date: 20-01-2011.

Bakonyi, T., Ivanics, E., Erdelyi, K., Ursu, K., Ferenczi, E., Weissenbock, H. & Nowotny, N. (2006) Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerging Infectious Diseases* **12**, 618-23.

Berthet, F.X., Zeller, H.G., Drouet, M.T., Rauzier, J., Digoutte, J.P. & Deubel, V. (1997) Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. *Journal of General Virology* **78,** 2293-2297.

Brummer-Korvenkontio, M., Vapalahti, O., Kuusisto, P., Saikku, P., Manni, T., Koskela, P., Nygren, T., Brummer-Korvenkontio, H. & Vaheri, A. (2002) Epidemiology of Sindbis virus infections in Finland 1981-96: possible factors explaining a peculiar disease pattern. *Epidemiology and Infection* **129,** 335-345.

Buckley, A., Dawson, A. & Gould, E.A. (2006) Detection of seroconversion to West Nile virus, Usutu virus and Sindbis virus in UK sentinel chickens. *Virology Journal* **3,** 71.

Busquets, N., Alba, A., Allepuz, A., Aranda, C. & Ignacio Nunez, J. (2008) Usutu virus sequences in Culex pipiens (Diptera: Culicidae), Spain. *Emerging Infectious Diseases* **14**, 861-863.

Calistri, P., Giovannini, A., Hubálek, Z., Ionescu, A., Monaco, F., Savini, G. & Lelli, R. (2010) Epidemiology of West Nile in Europe and in the Mediterranean basin. *Virology Journal* **4,** 29-37.

Calzolari, M., Bonilauri, P., Bellini, R., Albieri, A., Defilippo, F., Maioli, G., Galletti, G., Gelati, A., Barbieri, I., Tamba, M., Lelli, D., Carra, E., Cordioli, P., Angelini, P. & Dottori, M. (2010) Evidence of Simultaneous Circulation of West Nile and Usutu Viruses in mosquitoes sampled in Emilia-Romagna Region (Italy) in 2009. *PLoS ONE* **5,** 1-10.

Capobianchi, M.R., Sambri, V. Castilletti, C., Pierro, A. M., Rossini, G., Gaibani, P., Cavrini, F., Selleri, M., Meschi, S., Lapa, D., Di Caro, A., Grossi, P., De Cillia, C., Venettoni, S., Landini, M. P., Ippolito, G. & Nanni Costa, A. (2010) Retrospective screening of solid organ donors in Italy, 2009, reveals unpredicted circulation of West Nile virus. *Eurosurveillance* **15**.

Cavrini, F., Gaibani, P., Longo, G., Pierro, A.M., Rossini, G., Bonilauri, P., Gerundi, G.E., Di Benedetto, F., Pasetto, A., Girardis, M., Dottori, M., Landini, M.P. & Sambri, V. (2009) Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. *Eurosurveillance* **14**.

Chao, D.Y., Davis, B.S. & Chang, G.J. (2007) Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. *Journal of Clinical Microbiology* **45**, 584-9.

Dauphin, G., Zientara, S., Zeller, H. & Murgue B. (2004) West Nile: worldwide current situation in animals and humans. *Comparative Immunology, Microbiology and Infectious Diseases* **27**, 343-55.

Esteves, A., Almeida, A.P., Galao, R.P., Parreira, R., Piedade, J., Rodrigues, J.C., Sousa, C.A. & Novo, M.T. (2005) West Nile virus in Southern Portugal, 2004. *Vector Borne and Zoonotic Diseases* **5**, 410-3.

Fernandes, T., Clode, M.H.H., Simões, M.J., Ribeiro, H. & Anselmo, M.L. (1998) Isolation of virus West Nile from a pool of unfed *Anopheles atroparvus* females in the Tejo River estuary, Portugal. *Acta Parasitologica Portugal* **5**, 7.

Filipe, A. R. (1972) Isolation in Portugal of West Nile virus from *Anopheles maculipennis* mosquitoes. *Acta Virologica* **16**, 361.

Francy, D. B., Jaenson, T. G., Lundstrom, J. O., Schildt, E. B., Espmark, A., Henriksson, B., Niklasson, B. (1989) Ecologic studies of mosquitoes and birds as hosts of Ockelbo virus in Sweden and isolation of Inkoo and Batai viruses from mosquitoes. *American Journal of Tropical Medicine and Hygiene* **41,** 355-363.

Fyodorova, M. V., Savage, H.M., Lopatina, J.V., Bulgakova, T.A., Ivanitsky, A.V., Platonova, O.V. & Platonov, A.E. (2006) Evaluation of potential West Nile virus vectors in Volgograd region, Russia, 2003 (Diptera: Culicidae): species composition, bloodmeal host utilization, and virus infection rates of mosquitoes. *Journal of Medical Entomology* **43**, 552-63.

Ginsberg, H. S., Rochlin, I. & Campbell, S. R. (2010). The use of early summer mosquito surveillance to predict late summer West Nile virus activity. *Journal of Vector Ecology* **35,** 35-42.

Gould, E. A., Gallian, P., De Lamballerie, X. & Charrel, R.N. (2010) First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality! *Clinical Microbiology and Infection* **16,** 1702-1704.

Gratz, N.G. (2006) Vector- and Rodent-Borne diseases in Europe and North-America. Cambridge, United Kingdom, Cambridge University Press.

Hannoun, C., Panthier, R., Mouchet, J. & Ezouan, J. (1964) Isolation in France of the WNV from patients and from the vector *Culex modestus* Ficalbi. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* **259**, 4170-4172.

Higgs, S., Snow, K. & Gould, E.A. (2004) The potential for West Nile virus to establish outside of its natural range: a consideration of potential mosquito vectors in the United Kingdom. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**, 82-7.

Hubálek, Z. (2008) Mosquito-borne viruses in Europe. *Parasitology Research* **103** Suppl 1, S29-43.

Hubálek, Z. & Halouzka, J. (1996) Arthropod-borne viruses of vertebrates in Europe. *Acta Scientarum Naturalium Academiae Scientiarum Bohemicae Brno* **30**, 1-95.

Hubálek, Z., Rudolf, I., Bakonyi, T., Kazdova, K., Halouzka, J., Sebesta, O., Sikutova, S., Juricova, Z. & Nowotny, N. (2010) Mosquito (Diptera: Culicidae) surveillance for arboviruses in an area endemic for West Nile (Lineage Rabensburg) and Tahyna viruses in Central Europe. *Journal of Medical Entomology* **47,** 466-472.

Hubálek, Z., Wegner, E., Halouzka, J., Tryjanowski, P., Jerzak, L., Sikutova, S., Rudolf, I., Kruszewicz, A. G., Jaworski, Z. & Wlodarczyk, R. (2008) Serologic survey of potential vertebrate hosts for West Nile virus in Poland. *Viral Immunology* **21**, 247-253.

Jost, H., Bialonski, A., Schmetz, C., Gunther, S., Becker, N. & Schmidt-Chanasit, J. (2011) Isolation and Phylogenetic Analysis of Batai Virus, Germany. *American Journal of Tropical Medicine and Hygiene* **84,** 241-243.

Jost, H., Bialonski, A., Storch, V. Gunther, S., Becker, N. & Schmidt-Chanasit, J. (2010) Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. *Journal of Clinical Microbiology* **48,** 1900-1903.

Juriková, Z., Hubálek, Z., Halouzka, J. & Sikutová, S. (2009) Serological examination of songbirds (passeriformes) for mosquito-borne viruses Sindbis, Tahyna, and Batai in a South Moravian wetland (Czech Republic). *Vector-borne and Zoonotic Diseases* **9**, 295-299.

Karabatsos, N. (1985). International catalogue of arthropod-borne viruses. American Society for Tropical Medicine and Hygiene. San Antonio, Texas.

Katsarov, G., Vasilenko, S., Vargin, V., Burtenko, S. & Tkachenko, E. (1980) Serological studies on the distribution of some arboviruses in Bulgaria. *Problems Infectious Parasite Diseases* **8**, 32-35.

Koopmans, M., Martina, B., Reusken, C.B.E.M. & Van Maanen, K. (2008) West Nile virus in Europe. Chapter 8. *In:* Takken, W. & Knols, B. (Eds.) *Emerging pests and vector-borne diseases in Europe*. Wageningen Academic publishers.

Kramer, L.D., Li, J. & Shi, P.Y. (2007) West Nile virus. *Lancet Neurology* **6**, 171-81.

Kurkela, S., Manni, T., Myllynen, J., Vaheri, A. & Vapalahti, O. (2005) Clinical and laboratory manifestations of Sindbis virus infection: prospective study, Finland, 2002-2003. *Journal of Infectious Diseases* **191,** 1820-1829.

Kurkela, S., Ratti, O., Huhtamo, E., Uzcategui, N. Y., Nuorti, J. P., Laakkonen, J., Manni, T., Helle, P., Vaheri, A. & Vapalahti, O. (2008) Sindbis virus infection in resident birds, migratory birds, and humans, Finland. *Emerging Infectious Diseases* **14,** 41-47.

La Ruche, G., Souares, Y., Armengaud, A., Peloux-Petiot, F., Delaunay, P., Despres, P., Lenglet, A., Jourdain, F., Leparc-Goffart, I., Charlet, F., Ollier, L., Mantey, K., Mollet, T., Fournier, J. P., Torrents, R., Leitmeyer, K., Hilairet, P., Zeller, H., Van Bortel, W., Dejour-Salamanca, D., Grandadam, M. & Gastellu-Etchegorry, M. (2010) First two autochthonous dengue virus infections in metropolitan France, September 2010. Eurosurveillance **15,** 19676.

Labuda, M., Kozuch, O. & Gresikova, M. (1974) Isolation of West Nile virus from *Aedes cantans* mosquitoes in West Slovakia. *Acta Virologica* **18**, 429-433.

Linke, S., Niedrig, M., Kaiser, A., Ellerbrok, H., Muller, K., Muller, T., Conraths, F. J., Muhle, R. U., Schmidt, D., Koppen, U., Bairlein, F., Berthold, P. & Pauli, G. (2007) Serologic evidence of West Nile virus infections in wild birds captured in Germany. American Journal of Tropical Medicine and Hygiene **77,** 358-364.

Lukacik, G., Anand, M., Shusas, E.J. Howard, J.J., Oliver, J., Chen, H., Backenson, P.B., Kauffman, E.B., Bernard, K.A., Kramer, L.D. & White, D.J. (2006) West Nile virus surveillance in mosquitoes in New York State, 2000-2004. *Journal American Mosquito Control Association* **22,** 264-271.

Lundstrom, J.O. (1999) Mosquito-borne viruses in western Europe: a review. Journal of Vactor Ecology **24,** 1-39.

Lundstrom, J.O., Lindstrom, K.M., Olsen, B., Dufva, R. & Krakower, D.S. (2001) Prevalence of Sindbis virus neutralizing antibodies among Swedish passerines indicates that thrushes are the main amplifying hosts. *Journal of Medical Entomology* **38,** 289-297.

Norder, H., Lundstrom, J.O., Kozuch, O. Magnius, L.O. (1996) Genetic relatedness of Sindbis virus strains from Europe, Middle East, and Africa. Virology **222,** 440-445.

Papa, A., Danis, K., Baka, A., Bakas, A, Dougas, G., Lytras, T., Theocharopoulos, G., Chrysagis, D., Vassiliadou, E., Kamaria, F., Liona, A., Mellou, K., Saroglou, G. & Panagiotopoulos, T. (2010) Ongoing outbreak of West Nile virus infections in humans in Greece, July - August 2010. *Eurosurveillance* **15**(34).

Parreira, R., Severino, P., Freitas, F., Piedade, J., Almeida, A. P. & Esteves, A. (2007) Two distinct introductions of the West Nile virus in Portugal disclosed by phylogenetic analysis of genomic sequences. *Vector-borne and Zoonotic Diseases* **7,** 344-352.

Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabbatini, M.T., Tagliazucchi, S., Savini, G., Monaco, F., Simone, M.L., Lelli, R. & Rumpianesi, F. (2009) First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. *Eurosurveilance* **15**(50).

Poncon, N., Toty, C., L'Ambert, G., Le Goff, G., Brengues, C., Schaffner, F. & Fontenille, D. (2007) Biology and dynamics of potential malaria vectors in Southern France. *Malaria Journal* **6**, 18.

Rappole, J.H. & Hubálek, Z. (2003) Migratory birds and West Nile virus. *Journal of Applied Microbiology* **94** Suppl 47S-58S.

Rappole, J.H., Derrickson, S.R. & Hubálek, Z. (2000) Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerging Infectious Diseases* **6**, 319-28.

Reiter, P. (1983) A portable, battery powered trap for collecting gravid Culex mosquitoes. *Mosquito News* **43,** 496- 498.

Reusken, C., De Vries, A., Den Hartog, W., Braks, M. & Scholte E.J. (2010) A study of the circulation of West Nile virus in mosquitoes in a potential high-risk area for arbovirus circulation in the Netherlands, "De Oostvaardersplassen. *European Mosquito Bulletin* **28,** 69-83.

Reusken, C., De Vries, A., Buijs, J., Braks, M. A., den Hartog, W. & Scholte, E. J. (2010) First evidence for presence of Culex pipiens biotype molestus in the Netherlands, and of hybrid biotype pipiens and molestus in northern Europe. *Journal of Vector Ecology* **35,** 210-212.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M.G., Majori, G.C.& Cassone, A. (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370,** 1840-1846.

Sammels, L.M., Lindsay, M.D., Poidinger, M., Coelen, R. J. & Mackenzie, J.S. (1999) Geographic distribution and evolution of Sindbis virus in Australia. *Journal of General Virology* **80,** 739-748.

Sane, J., Guedes, S., Kurkela, S., Lyytikainen, O. & Vapalahti, O. (2010) Epidemiological analysis of mosquito-borne Pogosta disease in Finland, 2009. *Eurosurveillance* **15**(2).

Savage, H.M., Ceianu, C., Nicolescu, G., Karabatsos, N., Lanciotti, R., Vladimirescu, A., Laiv, L.,Ungureanu, A., Romanca, C. & Tsai, T.F. (1999) Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *American Journal of Tropical Medicine and Hygiene* **61**, 600-11.

Scholte, E., Den Hartog, W., Braks, M., Reusken, C., Dik, M. & Hessels, A. (2009) First report of a North American invasive mosquito species *Ochlerotatus atropalpus* (Coquillett) in the Netherlands, 2009. *Eurosurveillance* **14** (45).

Scholte, E., Den Hartog, W., Dik, M., Schoelitsz, B., Brooks, M., Schaffner, F., Foussadier, R., Braks, M. & Beeuwkes, J (2010) Introduction and control of three invasive mosquito species in the Netherlands, July-October 2010. *Eurosurveillance* **15**(45).

Scholte E.J., Dijkstra, E., Ruijs, H., Jacobs, F., Takken, W., Hofhuis, A., Reusken, C., Koopmans, M. & Boer de, A. (2007) The Asian tiger mosquito (*Aedes albopictus*) in the Netherlands: should we worry? *Proceedings of the Netherlands Entomological Society,* meeting **18**, 131-136.

Scholte, E.J., Dijkstra, E., Blok, H., De Vries, A., Takken, W., Hofhuis, A., Koopmans, M., De Boer, A. & Reusken, C. (2008) Accidental importation of the mosquito *Aedes albopictus* into the Netherlands: a survey of mosquito distribution and the presence of dengue virus. *Medical and Veterinary Entomology* **22**, 352-8.

Seidowski, D., Ziegler, U., von Ronn, J.A., Muller, K., Huppop, K., Muller, T., Freuling, C., Muhle, R., U., Nowotny, N., Ulrich, R. G., Niedrig, M. & Groschup, M.H. (2010). West Nile virus monitoring of migratory and resident birds in Germany. *Vector-borne and Zoonotic Diseases* **10,** 639-647.

Stevenson, J., Hymas, W. & Hillyard, D. (2008) The use of Armored RNA as a multi-purpose internal control for RT-PCR. *Journal of Virological Methods* **150,** 73-76.

Tamba, M., Bonilauri, P., Bellini, R., Calzolari, M., Albieri, A., Sambri, V., Dottori, M. & Angelini, P. (2010) Detection of Usutu Virus within a West Nile Virus Surveillance Program in Northern Italy. *Vector-borne and Zoonotic Diseases*. Epub ahead of print**.**

Taylor, R.M., Hurlbut, H.S., Work, T.H., Kingston, J.R., & Frothingham, T.E. (1955) Sindbis virus: a newly recognized arthropodtransmitted virus. *American Journal of Tropical Medicine and Hygiene* **4**, 844-62.

Vazquez, A., Sanchez-Seco, M.P., Ruiz, S., Molero, F., Hernandez, L., Moreno, J., Magallanes, A., Tejedor, C. G. & Tenorio, A. (2010) Putative new lineage of west nile virus, Spain. *Emerging Infectious Diseases* 1**6,** 549-552.

Verdonschot, P. (2002). Culicidae. pp. 98-100. *In*: Beuk, P.L.Th. (ed.). *Checklist of the Diptera in the Netherlands*. KNNV publishers, Utrecht, the Netherlands.

Weaver, S. C. and W. K. Reisen (2010) Present and future arboviral threats. *Antiviral Research* **85,** 328-345.

Weissenbock, H., Hubálek, Z., Bakonyi, T. & Nowotny, N. (2009) Zoonotic mosquito-borne flaviviruses: Worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. *Veterinary Microbiology* **140**, 271-280.

Weissenböck, H., Chvala-Mannssberger, S., Bakonyi, T. & Nowotny, N. (2008) Emergence of Usutu virus in Central Europe: diagnosis, surveillance and epizootology. In: *Emerging pests and vectorborne diseases in Europe*. Takken, W. & Knols, B. (Eds.) Wageningen Academic publishers.

Williams, G. M. & J. B. Gingrich (2007) Comparison of light traps, gravid traps, and resting boxes for West Nile virus surveillance. *Journal of Vector Ecology* **32,** 285-291.