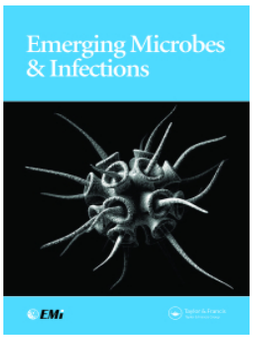

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Sentinel chicken surveillance reveals previously undetected circulation of West Nile virus in the Netherlands

Kiki Streng[a]#, Nnomzie Atama[b]#, Felicity Chandler[b], Rody Blom[c], Henk van der Jeugd[d], Maarten Schrama[e], Marion P.G. Koopmans[b], Wim H.M. van der Poel[a,f] and Reina S. Sikkema [b,d]

[a]Quantitative Veterinary Epidemiology, Wageningen University and Research, Wageningen, the Netherlands; [b]Viroscience, ErasmusMC, Rotterdam, the Netherlands; [c]Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands; [d]Dutch Centre for Avian Migration and Demography, NIOO – KNAW, Wageningen, the Netherlands; [e]Institute of Environmental Sciences, Leiden University, Leiden, the Netherlands; [f]Wageningen Bioveterinary Research, Lelystad, the Netherlands

ABSTRACT

West Nile virus (WNV) was first detected in the Netherlands in 2020, with circulation observed in birds, mosquitoes, and humans in two geographical areas. Usutu virus (USUV) has been circulating in the Netherlands since 2016. Following the detection of WNV in the Netherlands, we investigated the possible use of petting zoos as urban sentinel sites to examine the extent of WNV and USUV circulation around the two WNV outbreak locations. Chickens at petting zoos and in backyards were sampled within a 15-kilometer radius of the confirmed WNV circulation areas at three timepoints over one year (2021–2022). Sera were analysed using a protein microarray for binding antibodies to orthoflavivirus NS1 antigens and reactive samples were confirmed through micro-focus reduction neutralization tests (mFRNT). Furthermore, mosquitoes at sampling locations were collected to assess their blood feeding behaviour. This serosurvey detected the circulation of USUV and WNV in petting zoo and backyard chickens in 2021, both within and outside the 2020 outbreak areas. The WNV circulation was not detected by other existing surveillance schemes in mosquitoes, wild birds, horses and humans. In addition, the results show rapid decay of USUV antibodies in approximately 20 weeks. Our findings support the utility and the added value of petting zoo chickens as sentinels for monitoring USUV and WNV circulation compared to other available methods. Seroconversions observed in petting zoos and backyard chickens living in or near densely populated urban areas further highlighted potential public health risks that went undetected.

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KEYWORDS West Nile virus; Usutu virus; Orthoflavivirus; chicken; sentinel; serology; surveillance

Introduction

West Nile virus (WNV) and Usutu virus (USUV) are both zoonotic mosquito-borne viruses in the Japanese encephalitis serogroup and belong to the *Flaviviridae* family and genus *Orthoflavivirus* [1]. Both viruses are maintained in an enzootic transmission cycle between birds and mosquitoes (primarily *Culex* mosquitoes) and are known to co-circulate in parts of Europe [2–4]. *Culex pipiens* is recognized as the primary vector for WNV and USUV in Europe. In the Netherlands, *Cx. pipiens* is a ubiquitous and highly abundant mosquito species [5–7]. *Culex pipiens* are known to feed both on avian and mammalian hosts including humans, which may facilitate the spread and spillover of USUV and WNV [8].

The Usutu virus has been circulating in continental Europe for more than two decades [9]. In the Netherlands, USUV was detected in 2016 for the first time and has caused significant outbreaks in birds, with associated mortality specifically in wild blackbirds (*Turdus merula*) and captive owls (Strigiformes) from 2016 to 2018 [10,11]. In 2018, a study on Dutch blood donors revealed multiple (asymptomatic) human USUV infections, which occurred concurrently with an observed increase in bird mortality in the study area [12]. Surveillance of live and dead wild birds and mosquitoes has shown ongoing circulation of USUV in the years after [13].

Over the last decades, WNV has become one of the most widespread arboviruses in the world [14]. Outbreaks of disease caused by WNV have been extensively described in southern Europe. In 2018 a major outbreak resulted in 1311 confirmed human cases across Europe [15]. In the same year, the virus also was detected northwards as the first WNV cases in Germany were observed in birds and horses [16]. In August 2020,

CONTACT Reina S. Sikkema r.sikkema@erasmusmc.nl
#Authors contributed equally.

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the virus was detected for the first time in the Netherlands in a common whitethroat (*Curruca communis*) and *Culex* mosquitoes [17]. In October of the same year, the first autochthonous case of human WNV neuroinvasive disease was identified in the Netherlands. Retrospective analysis revealed six additional clinical cases [18]. All WNV detections within the Netherlands were restricted to two specific areas; in the municipality of Utrecht where most WNV positive birds and all positive mosquitoes were found; and near the municipality of Arnhem where a seventh human case was identified in October [19,20].

According to the World Organisation for Animal Health (WOAH), sentinels are defined as susceptible animals of known health or immune status that are regularly tested in specific (outbreak-prone) geographical locations to detect the occurrence of diseases or infections, often through serological testing [21]. Captive sentinel birds, such as chickens (*Gallus gallus domesticus*) and pigeons (*Columbia livia*) have been routinely utilized for arbovirus monitoring and surveillance in various settings and across different continents [22–24]. Chickens do not show clinical signs following infection but do develop neutralizing antibodies. Experimental studies have shown that chickens do not contribute to the vector-host transmission cycle [25,26]. In addition, sentinels such as chickens can be repeatedly sampled at the same desired locations, while also leveraging on their historical data on origin and movement patterns. Hence, chickens can serve as an effective sentinel model system for the early detection or enzootic transmission of WNV and USUV.

Similar to other European countries, the Netherlands has a high density of petting zoos (also called city farms or urban farms), which are often located in peri-urban and urban areas [27]. Of the about 500 petting zoos in the country, 90% keep chickens alongside other animals, such as peacocks, sheep and goats [27]. Petting zoos may provide an innovative and sustainable approach to sentinel surveillance for orthoflaviviruses, considering that these zoos are usually park-like structures in areas with relatively high human population densities. Mosquito-borne virus detection in petting zoos may therefore provide an indication of the risk of spill-over or concurrent circulation in the human population [28].

Although WNV surveillance in humans, animals and vectors was increased following its first detection in the Netherlands, the geographical spread of WNV around the outbreak areas after the first detections remained unknown. We therefore studied the spread of the virus in both outbreak areas, using chickens in petting zoos as sentinels. In addition, mosquitoes were collected to assess the presence of competent WNV and USUV vectors and their blood-feeding patterns. By employing these approaches, we explored the potential of petting zoos as sentinel sites for monitoring USUV and WNV in the Netherlands.

Materials and methods

Sampling

Chickens from petting zoos and backyards within a 15 km radius of each of the two WNV outbreak locations [17,19,20] were included for sampling. Twenty-three locations around Utrecht and 13 around Arnhem agreed to participate (Figure 1). Additionally, volunteer bird ringers collected samples from backyard chickens between October 2020 and June 2022, from within and outside of our two specified study areas and radius (see Figure 1). The sampling of chickens was conducted in three phases: 1st of October 2020–31st of May 2021 (in both Utrecht and Arnhem), 1st of June – 31st of October 2021 (Utrecht area only) and 1st of November 2021 – 1st of June 2022 (both Utrecht and Arnhem), which we refer to in this study as phases I, II, and III respectively. Chickens were individually ringed for identification. If possible, individual chickens were resampled throughout the sampling phases. However, some chickens were lost to follow-up, because they died or were relocated away from the study area. These chickens were replaced by new chickens if available at the same location. A minimum of two and a maximum of thirteen chickens were sampled at each location per timepoint. Blood was obtained from the cutaneous ulnar vein using a syringe and needle. Blood samples were transported to the laboratory and then centrifuged at 13,000 rpm for 5 min to collect sera, which were stored at – 80°C until use.

In addition, mosquitoes were collected to assess the presence of competent WNV and USUV vectors and their blood-feeding patterns in the petting zoos. Mosquitoes were collected during phases II and III at sampling locations using manual aspirators. All chicken coops, canteens and barns on the premises were visually inspected for the presence of mosquitoes. Mosquitoes were aspirated and stored frozen in 50 mL falcon tubes at −20°C until identification and further processing at the laboratory.

Laboratory analysis

Protein microarray

All sera with sufficient volume were tested on a protein microarray (PMA) as previously described [29]. In brief, each NS1 antigen (USUV, The Native Antigen Company, Kidlington UK; and WNV, Sino biologicals, China) was spotted in duplicate onto the nitrocellulose pad-coated glass slide (Sartorius Stedim Biotech, Goettingen, Germany). Slides were incubated with sera after blocking with a Blocker™ BLOTTO buffer in TBS (Thermo Scientific, Rockford, IL, USA) to minimize aspecific binding, and subsequently with Alexafluor-647 conjugated goat anti-chicken IgY (Jackson

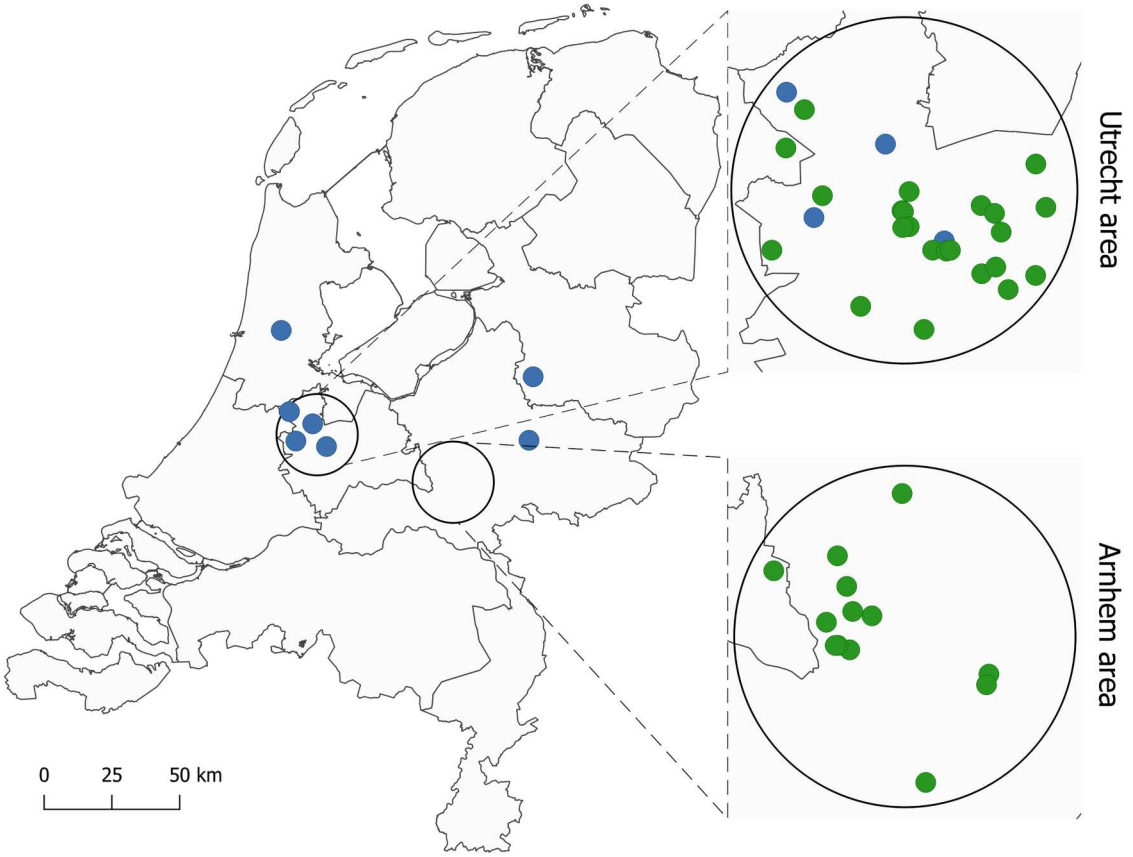

Figure 1. Sampling locations (green dots) within the 15 km radius of two locations where WNV was detected in 2020. Locations where backyard chickens were sampled by volunteer bird ringers are shown in the full country map and the Utrecht area inset as blue dots.

ImmunoResearch, Inc., West Grove, USA). Each serum was tested in a 4-fold serial dilution ranging from 1:20–1:1280. The tested microarray slides were scanned using a PowerScanner™ (Tecan, Männedorf, Switzerland), and the relative fluorescence units (RFU) per antigen were analysed using ImaGene® software (Biodiscovery, El Segundo, USA). Titers were calculated from the average RFU values using GraphPad Prism vs 9.4.0, as previously described [30].

Protein microarray cut-offs for chickens were calculated using a ROC curve, using antibody titers of: samples collected from chickens in the Netherlands in 2006 before the first Dutch detection of USUV; Specific Pathogen Free (SPF) chickens; PCR-confirmed USUV and WNV positive birds, and chicken sera collected during this study. The cut-off with the highest specificity for USUV and WNV (Supplemental material A, S1) was selected. The cut-off value for the PMA USUV NS1 and WNV NS1 signals was estimated at a median fluorescence value of ≥3500 at serum dilution 1:80.

Micro-focus reduction neutralization test (mFRNT)

Samples with antibody binding signals above cut-off for USUV or WNV on the PMA were selected for further determination of the presence of neutralizing antibodies against WNV (lineage 2; B956, NCPV Porton Down #638, 2010) and USUV (Africa 3 strain; Turdus Merula NL isolate 2016, EVAg Ref-SKU: 011V-02153) using a micro-focus reduction neutralization test (mFRNT) as previously described [31]. The positivity cut-off specific to chickens was established at ≥160 for USUV and ≥80 for WNV. Samples showing positivity for both USUV and WNV mFRNTs, with a < 4-fold titer difference between the two antigens are denoted as "FLAVI" i.e. orthoflavivirus-positive (see Supplemental material A, S2). An overview of all mFRNT titers for all tested chickens is also shown in the Supplementary material A (S3).

Virus neutralization test (VNT)

Six samples from phase I were confirmed by Virus Neutralization Tests (VNT) instead of mFRNT because of insufficient volume to retest those in the mFRNT. Virus neutralization tests were performed using titrated stocks of USUV (Africa 3 strain; Turdus Merula NL isolate 2016, EVAg Ref-SKU: 011V-02153) and WNV (lineage 2, strain B956, NCPV Porton Down #638, 2010), using a protocol adapted from Reusken et al. [32]. Viral cytopathic effects (CPE) were recorded five days post-inoculation for USUV and seven days post-inoculation for WNV. Sera were regarded positive in the case of a reciprocal titer of

≥1:16 and a ≥ 4-fold titer difference was used to distinguish between WNV and USUV infections.

Statistical analysis and mapping

Descriptive statistics were used to summarize the antibody detections across sampling locations and areas. Individual and flock seroprevalence of WNV and USUV was estimated as the proportion of seropositive individuals/locations to the total number sampled per location/area, using a two-sided exact binomial test with a 95% confidence interval. Waning of antibodies over time was estimated by comparing mFRNT titers across phases using a mixed-effect linear model using the lme4 package in RStudio [33]. The dependent variable was the log-transformed mFRNT titer, while the fixed factor was the number of weeks after the first positive sample. To account for the repeated sampling on the same individual, we included individual chicken identification number as a random factor. For this model, we selected chickens with an USUV/WNV status in phase I or II and that were sampled in more than one phase. Analyses included only those results from the USUV/WNV status and onward. Chickens showing an increase in titer between sampling points were removed to exclude potentially reinfected animals. All data handling, statistical analyses, and graphs were generated using R statistical software vs4.1.2. Maps were created using QGIS desktop version 3.22.5 [34].

Mosquito and bloodmeal host identification

Mosquitoes were identified to the species level following the identification key of Becker et al. [35]. Adult female *Cx. pipiens* and *Cx. torrentium* are morphologically indistinguishable and were therefore grouped together as *Cx. pipiens/torrentium*. Subsequently, blood-engorged specimens were subjected to bloodmeal analysis following the molecular protocol described by Blom et al. [36]. In brief, DNA was extracted from individual blood-engorged abdomens, followed by PCR. PCR was conducted with primer sets targeting the Cytb region. In case amplification with Cytb primers was unsuccessful, an additional PCR was performed using primers targeting the 16S rDNA region. Successful PCR products were subjected to Sanger sequencing. Sequences were analysed using Geneious Prime 2023.0.4. Acquired sequences were matched with reference sequences in the NCBI Genbank database using BLAST to identify the host origin.

Results

Samples and resampling of chickens

Sampling of chickens was performed in three phases between October 2020 and June 2022. In total, we collected 639 sera from 348 individual chickens across 36 locations that were within a 15 km radius of the two 2020 WNV outbreak locations in the Netherlands (see Table 1). Two samples from the Utrecht area had insufficient volume and thus were not tested. Additionally, volunteer bird ringers collected 31 samples from 30 chickens at seven locations during the study period, four of which were within the 15 km radius in Utrecht (see Figure 1). Twenty-four samples (19 from the Utrecht area and five ringer samples) were positive on PMA but had insufficient volume left to be tested by mFRNT and were excluded from the analyses (see Table 1).

Of the 370 chickens tested, seventy-six chickens (20.5%) were sampled and tested three times (all in Utrecht), 122 (33.0%) chickens twice (55 Utrecht and 66 Arnhem, 1 ringer chicken) and 172 (46.5%) were sampled and tested only once (109 Utrecht and 39 Arnhem, 24 ringer chickens).

Serology

Seroprevalence and seroconversions in individual chickens

In the Utrecht area, WNV seroprevalence showed fluctuations across the three phases: 3.33% [6/180 (95% CI: 1.23, 7.11)] in phase I, 13.29% [15/143 (95% CI: 8.19, 19.96)] in phase II, and 12.10% [15/124 (95% CI: 6.93, 19.17)] in phase III. In contrast, USUV seroprevalence increased steadily from 6.67%

Table 1. Numbers of tested locations and chicken samples per phase, per area.

Period	Phase I (1 Oct 2020–31 May 2021)			Phase II (1 June 2021–31 Oct 2021)		Phase II (1 Nov 2021–1 June 2022)		
Area	Utrecht	Arnhem	Ringer locations	Utrecht	Ringer locations	Utrecht	Arnhem	Ringer locations
Number of locations[†]	23	13	6	20	1	21	13	2
New chickens sampled	180	96	15	35	4	13	9	6
Resampled	–	–	–	112	0	113	66	1
Lost to follow-up	-	-	-	*83*	*15*	*42*	*30*	*3*
Insufficient volume	*15*	*0*	*5*	*4*	*0*	*2*	*0*	*0*
Total fully tested (sampled) per area	180 (195)	96 (96)	15 (20)	143 (147)	4 (4)	124 (126)	75 (75)	7 (7)
Total tested (sampled) per phase	290 (311)			147 (151)		206 (208)		

[†]Two locations from the Utrecht area were lost to follow-up due to an avian influenza outbreak and one location not being interested in further participation. One location from the Utrecht area had all chickens relocated and was followed up at the new location as this was still within the sampling radius. One location from the Utrecht area only participated in phases I and III.

[12/180 (95% CI: 3.49, 11.36)] in phase I to 10.49% [15/143 (95% CI: 5.99, 16.71)] in phase II, and rose again to 20.97% [26/124 (95% CI: 14.18, 29.19)] in phase III. Arnhem area had lower WNV seroprevalences of 1.04% [1/96 (95% CI: 0.03, 5.67)] in phase I and 6.67% [5/75 (95% CI: 2.20, 14.88)] in phase III compared to Utrecht. Conversely, the USUV seroprevalence in the Arnhem area was 20.83% [20/96 (95% CI: 13.20, 30.33)] in phase I surpassing the seroprevalence in Utrecht. However, by phase III the USUV seroprevalence in Arnhem dropped to 8.0% [6/75 (95% CI: 2.99, 16.60)]. No sampling was conducted in the Arnhem area in phase II, hence the seroprevalence was not estimated (see Table 2; Figure 2).

Three out of seven volunteer bird ringer locations were located outside the two investigated areas. In phase I, in a location 40 km north-west of Utrecht, one of two chickens tested WNV positive by VNT. In another location 22 km north-east of Arnhem, one of two chickens tested seropositive for WNV. In phase II the same location was sampled, and one (1/4) newly sampled chicken tested WNV positive. In phase III, the same location had one new (1/4) WNV-positive and one (1/4) USUV-positive chicken. All other positive ringer sampled chickens, sampled in phase I, were in the 15 km radius of the Utrecht area (see Figure 2A).

In total, 52 individual chickens seroconverted during our study period. Of these, 45 were from the Utrecht area and the remaining seven from the Arnhem area. Of the chickens from the Utrecht area that were sampled in the first two phases, 18.37% (18/98) seroconverted. These chickens developed antibodies against WNV (7/18), USUV (7/18) and both viruses (FLAVI, 4/18) indicating active circulation of both WNV and USUV in the summer of 2021 (1st of March-10th of October). Twenty-one chickens from Utrecht seroconverted between phase II and III (27th of September 2021-7th of March 2022) and developed antibodies against WNV (9/21), USUV (8/21), and both viruses (FLAVI, 4/21). In addition, 6 chickens from Utrecht seroconverted for WNV (1/6), USUV (4/6) and FLAVI (1/6) between phase I and III (not sampled during phase II). Seven chickens from the Arnhem area seroconverted for WNV (3/7), USUV (3/7) and FLAVI (1/7) between phase I and III.

Observed flock prevalences

The observed WNV flock (petting zoo) prevalence also showed a marked increase across the first two phases in the Utrecht area, from 21.74% [5/23, (95% CI: 7.46, 43.70)] to 60% [12/20, (95% CI: 36.05, 80.88)] (see Figure 2). After phase II, the flock prevalence in Utrecht remained stable at 52.38% [11/21, (95% CI: 29.78, 74.29)] in phase III. There was only one WNV-positive flock in the Arnhem area in phase I, thus a 7.69% [1/13, (95% CI: 0.19, 36.03)] flock prevalence, which increased to 23.08% [3/13, (95% CI: 5.04, 53.81)] in phase III. The observed USUV flock prevalence increased from 39.13% [9/23, (95% CI: 19.71, 61.46)] in phase I to 50% [10/20, (95% CI: 27.20, 72.80)] in phase II and 52.38% [11/21, (95% CI: 29.78, 74.29)] in phase III in the Utrecht area. In the Arnhem area, USUV flock prevalence decreased from 46.15% [6/13, (95% CI: 19.22, 74.87)] in phase I to 23.08% [3/13, (95% CI: 5.04, 53.81)] in phase III.

Antibody waning

Eight animals remained seropositive for either WNV ($n = 1$), USUV ($n = 4$) or both viruses ($n = 3$), throughout the complete study period of one year. These findings can indicate antibody persistence or reinfections in these animals. Forty-one chickens seroreverted from WNV ($n = 11$), USUV ($n = 25$) or

Table 2. Summary of percentage antibody positives across three sampling phases (I, II, & III) and sampling areas (Arnhem, Utrecht, and ringer locations).

	Phase I			Phase II			Phase III		
	Protein Array	mFRNT / VNT		Protein Array	mFRNT		Protein Array	mFRNT	
Area	% pos (d/N)	Antigens	% pos (d/N)	% pos (d/N)	Antigens	% pos (d/N)	% pos (d/N)	Antigens	% pos (d/N)
		WNV	1.61 (1/62)		WNV	NA		WNV	12.5 (5/40)
Arnhem	64.6 (62/96)	USUV	32.4 (20/62) ǂ[1]	NA	USUV	NA	53.3 (40/75)	USUV	15 (6/40)
		FLAVI	4.8 (3/62)		FLAVI	NA		FLAVI	2.5 (1/40)
		WNV	8.8 (6/68)		WNV	22.6 (19/84)		WNV	22.7 (15/66)
Utrecht	42.3 (82/194)	USUV	17.6 (12/68)	59.9 (88/147)	USUV	17.9 (15/84)	55.2 (69/125)	USUV	39.4 (26/66)
		FLAVI	10.3 (7/68)		FLAVI	8.3 (7/84)		FLAVI	9.1 (6/66)
		WNV	30.8 (4/13) ǂ[4]		WNV	25 (1/4)		WNV	14.3 (1/7)
Ringer locations	65.0 (13/20)	USUV	15.4 (2/13) ǂ[2]	100 (4/4)	USUV	0 (0/4)	100 (7/7)	USUV	14.3 (1/7)
		FLAVI	7.7 (1/13) ǂ[1]		FLAVI	0 (0/4)		FLAVI	0 (0/7)
Overall positivity	50.6 (157/310)		42.8 (62/145) ǂ	60.9 (92/151)		47.7 (42/88)	56.0 (116/207)		53.0 (61/115)

ǂ[n] Indicates samples (n = number of samples) confirmed via VNT instead of mFRNT. NB. Some of the PMA positives could not be confirmed on FRNT or VNT due to insufficient volume of sera.

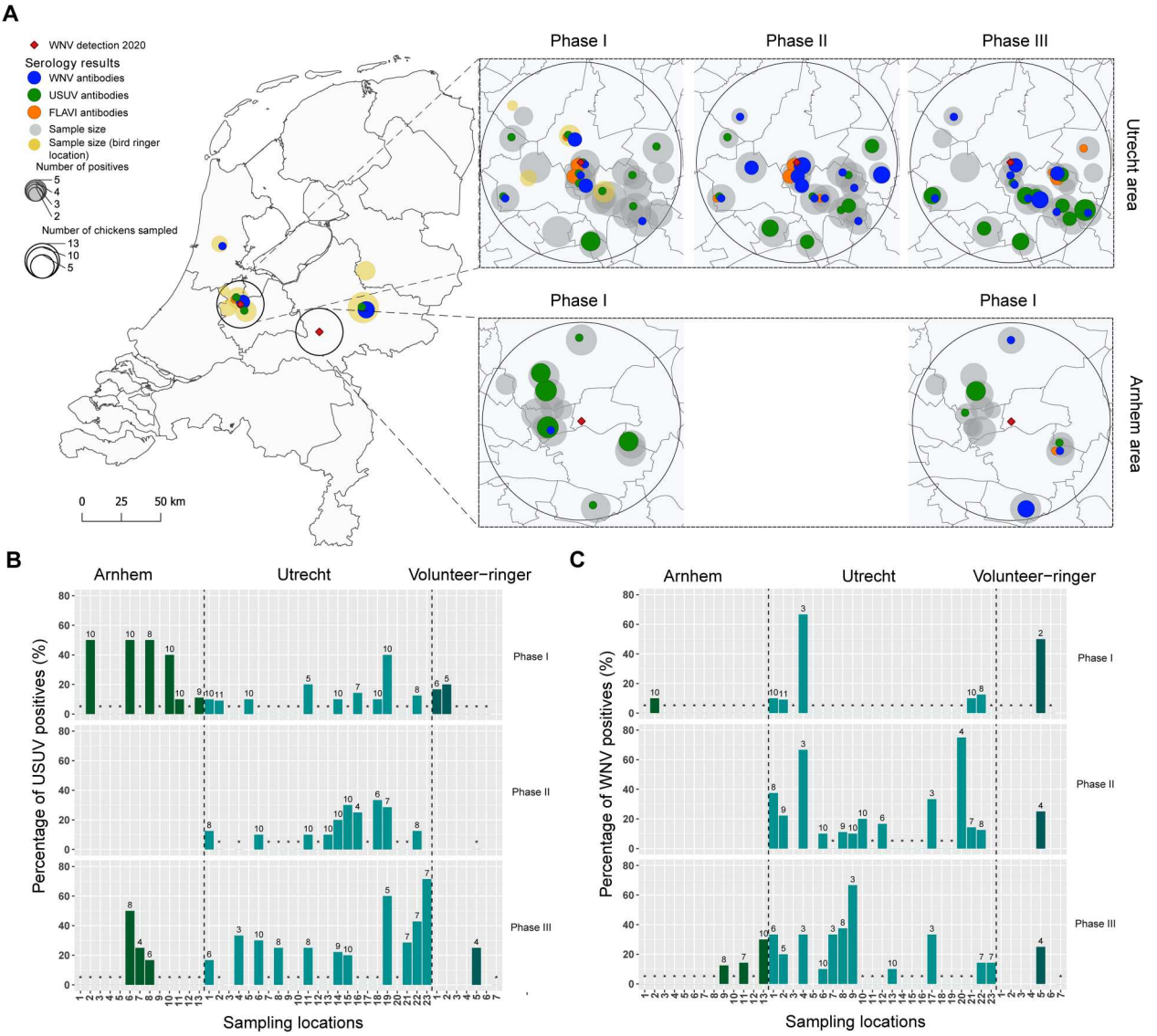

Figure 2. A: Spatial overview of all serological results for Utrecht and Arnhem area per sampling phase. Results for volunteer bird ringer locations outside of the two sampling areas ($n = 3$), aggregated for all phases, are shown in the large left (country) panel. B: Percentage of USUV-positive chickens per location for each sampling phase. C: Percentage of WNV-positive chickens per location for each sampling phase. *Indicates samples were taken but none were positive. Numbers above bars represent number sampled per location.

FLAVI ($n = 5$) seropositive to negative between sampling points. Twenty-seven out of 68 USUV positive chickens were repeatedly sampled and had an USUV positive status in phase I or II (without an increase in titer throughout the study period) and were selected to study waning of antibodies (see Figure 3). The intercept of the linear mixed-effects model was 6.07, which corresponds to an average estimated mFRNT titer of 432.7 for USUV at the first positive sampling. The model revealed an estimated average decline in log-transformed USUV titer of 0.049 per week since the first USUV status sample. This means the mFRNT titer of chickens would fall below cut-off (titer of 160) in about 20 weeks and below a titer of 10 (undetectable titer) in approximately 78 weeks. The sample size ($n = 13$) for waning of WNV antibodies was insufficient to perform a similar analysis. Four of these thirteen chickens had stable WNV titers above cut-off (80, 80, 1280 and 2560 respectively) at all sampling points, thus no waning of antibody titers, over a period ranging from 115 and 439 days between first and last sampling. Seven chickens had a ≥ 4-fold decline in WNV titer (with 120–347 days between first and last sample) and the remaining two had a 2-fold decline between the first and last sample (208 and 347 days respectively). In total, eight (8/13) of these chickens seroconverted.

Mosquitoes

In total, 47 mosquitoes were captured during visits in phase II ($n = 36$) and phase III ($n = 11$) at 12 different sampling locations. Both female (40/47, 85.1%) and male (7/47, 14.9%) mosquitoes were caught. The majority were *Culex* mosquitoes (59.6%) of which all but two were *Culex pipiens/torrentium*. The remaining

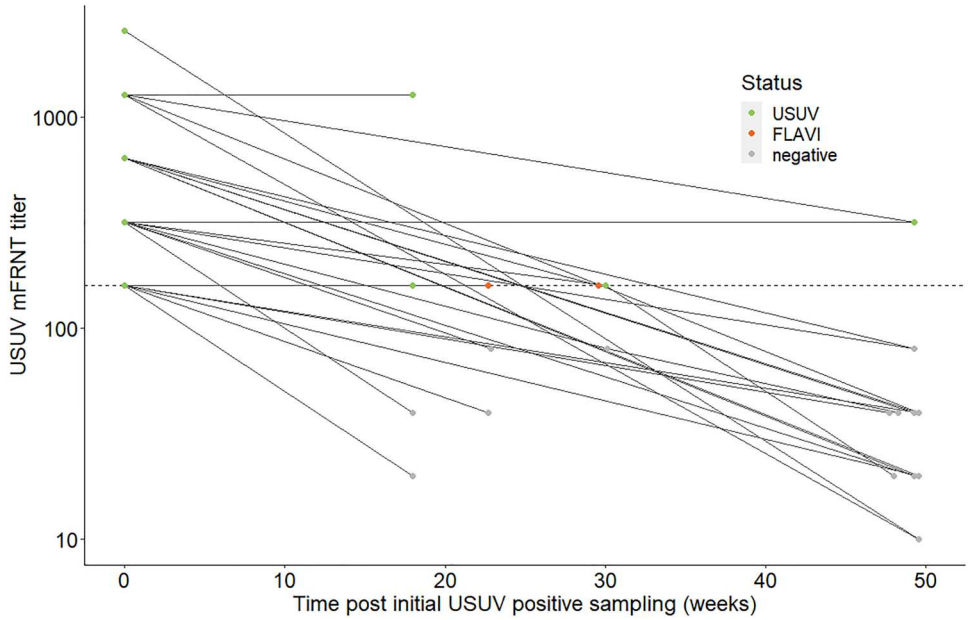

Figure 3. USUV mFRNT titers for 27 repeatedly sampled chickens confirmed positive for USUV in phase I or phase II. The horizontal dashed line indicates mFRNT cut-off titer for USUV. Status at each sampling point is indicated by a coloured dot.

two (male) *Culex* specimens were not identifiable to the species level. Other detected species were *Anopheles maculipennis s.l.* (23.4%), *Anopheles claviger* (4.3%) and *Culiseta annulata* (12.8%). Fifteen (15/40, 37.5%) of the female mosquitoes were engorged at the time of capture. The engorged females were all caught during phase II in September 2021 at five separate locations in the Utrecht area. Bloodmeal analyses revealed ten (all *Cx. pipiens/torrentium*, 66.7%) of the blood-engorged mosquitoes fed on chickens (*G. gallus*) and three mosquitoes (20%, two *An. claviger*, one *Cs. annulata*), all from the same farm, fed on pigs (*Sus scrofa*). For the remaining two blood-engorged mosquitoes, the source of the bloodmeal could not be identified.

Discussion

This study investigated the potential of utilizing petting zoo and backyard chickens as sentinels to assess the extent of spread and circulation of WNV and USUV in two WNV outbreak areas in the Netherlands. Through systematic sampling of chickens around outbreak sites, previously undetected transmission of WNV and USUV in the Netherlands was captured in 2021. This underscores the utility of chickens in backyard and petting zoo settings as effective sentinels for detecting the presence of WNV and USUV.

The observed prevalence of WNV at both individual and flock level in chickens around Utrecht and Arnhem showed an increase after May 2021, indicating active circulation of the virus during that year. This was corraborated by seroconversions in repeatedly sampled individuals across multiple locations. Furthermore, the detection of WNV antibodies in chickens located farther from the outbreak locations suggests a broader spread of the virus compared to areas identified through molecular surveillance in wild birds [17]. Notably, WNV circulation in 2021 went unnoticed by syndromic surveillance in horses and humans, as well as in molecular surveillance of mosquitoes and wild birds. However, the circulation of WNV in the Netherlands was later further affirmed by the detection of virus in a wild-caught grey heron (*Ardea cinerea*) in 2022. The partial sequence obtained from this bird clustered with the 2020 WNV sequences from the Netherlands [37]. In addition, USUV antibodies were consistently found in chickens and seroconversions were observed throughout the study period. Similar to the findings on WNV, this indicates active USUV circulation in both study areas. These findings corroborate the endemic nature of USUV in the Netherlands, supported by the annual detections in wild birds since 2016 (Münger et al., in prep). Surprisingly, no notable increase in blackbird mortality was observed in 2021 [38]. Our findings underscore the significance of integrating chicken serological surveillance into exisiting surveillance efforts for early-detection and response to USUV and WNV outbreaks.

Research on antibody waning after orthoflavivirus infections in animals is limited. Sentinel chickens are typically removed post-seroconversion, while recapturing wild birds is rare, complicating antibody decay tracking [23]. Experimental infection studies often do not study long-term antibody kinetics [25]. Our study estimates an average antibody decay to below cut-off at approximately 20 weeks post-initial positive sample. However, individual variation in

neutralizing antibody development and persistence exists [24]. Our findings align with a study in captive birds of prey showing a marked USUV antibody decline over six months [39]. However, Bergmann et al. [40] reported prolonged USUV positivity in zoo birds over four years, suggesting possible differential responses to orthoflavivirus infections between bird species. In our study, limited sample size hindered reliable analyses of WNV antibody waning, but some animals maintained stable antibody titers beyond 439 days post-initial sampling. Compared to USUV, indeed longer WNV antibody persistence was previously reported in various bird species, [41–44]. However, in none of these studied chickens were investigated.

A few seropositive chickens showed increasing titers over time, possibly due to new exposures or co-infections with other orthoflaviviruses [45]. These factors are not easily discernible in field investigations. Some chickens maintained persistent high antibody titers throughout the study, suggesting either reinfection or a prolonged half-life of antibodies due to individual variation. Alternatively, chronic WNV infections may have led to recrudescence [46,47]. In the case of USUV infections in 2020, chickens might have been infected months or even longer before the first sample was taken in early 2021, leading to potential underestimation of USUV-positive chickens and lower the expected timespan to go below cut-off.

Apart from chickens, horses, dogs and other wildlife species are commonly used as sentinels for orthoflavivirus surveillance [48–50]. However, horses may be vaccinated against WNV, rendering these individuals unsuitable as sentinels. In the Netherlands, WNV and USUV seroprevalence in horses and dogs is very low and equine cases of WNV infections have not been detected prior to human cases [51]. Obtaining wildlife samples is challenging, and repeated sampling is often impractical [50]. Furthermore, seroconversions in wildlife might not be the best reflection of public health risks as wildlife habitat are often segregated from urban areas. In contrast, chickens are logistically easier to procure, monitor and replace, enhancing their applicability as sentinel species. In addition, petting zoos and backyard chickens, often located in or near urban areas, may reflect human health risks related to WNV and USUV as shown previously [52]. The detection of chicken DNA in the bloodmeals *Cx. pipiens/torrentium* further confirms that chickens in petting zoos are exposed to bites of an important WNV and USUV vector [4,53]. Surveillance programmes incorporating repeated sampling of sentinel animals, like chickens, are better poised to capture temporal changes in virus activity viral for timely and effective public health interventions compared to cross-sectional studies.

In summary, this study provides strong evidence of the active circulation of both WNV and USUV throughout our study period, extending well beyond previously documented geographical detection range in the Netherlands. This also indicates that the total geographical range of WNV and USUV circulation is likely even larger, necessitating studies including broader perimeters from initial infection sites. Notably, no human cases were reported in the Netherlands during or after the study period. However, given the high antibody prevalence in chickens, our findings suggest the possibility of undetected human infections when relying solely on molecular and syndromic surveillance.

Overall, our study underscores the value of sentinel surveillance in petting zoos and backyard chickens in detecting virus circulation that might otherwise go unnoticed. Additionally, it affirms the utility of chickens as sentinels, complementing other surveillance methods such as wild bird and mosquito surveillance, as well as syndromic surveillance in horses and humans. Further insights into human infections could be gained through retrospective testing of bio-banked blood donors or hospitalized patient samples with a history of fever and/or neurological symptoms, collected from risk areas and during outbreak periods.

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Ethical statement

Sampling of chickens was performed in accordance with the Dutch law on Animal Experiments (WoD) and EU regulation on the use of animals for scientific purposes under document numbers AVD801002015342 and AVD-80100202114410.

ORCID

Reina S. Sikkema http://orcid.org/0000-0001-7331-6274

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