Supplementary Information for

One Health approach uncovers emergence and dynamics of Usutu and West Nile viruses in the Netherlands

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Supplementary Methods

Animal Sampling, Pre-Diagnostic Processing and Analyses

For live landbirds and waterbirds, viral diagnostics were performed by RT-PCR on throat and cloacal swabs, with unconfirmed detections occasionally verified using a second sample type. Most waterbirds were tested only using molecular methods. Based on species and capture histories, birds with evidence of West-Nile virus exposure were classified as local, non-local, or unknow. Non-migratory, resident species were always considered local. Migrant species that are present in the Netherlands only in either summer or winter were always considered non-local. Blackbirds, which both breed and winter in the Netherlands were considered local if sampled outside the migration and winter season, or when additional captures of the same individual indicated that the bird was part of the local resident breeding population. In other cases, birds were categorized as unknown. Between 2016 and 2018, following initial detections of USUV related mortality, dead free-ranging birds were preferentially selected for post-mortem investigation at locations where USUV activity had not been identified before. Dead captive birds were submitted for necropsy at the Veterinary Pathology Diagnostic Centre (VPDC). A detailed description of necropsies is provided in Giglia et al[1]. Testing for USUV and WNV diagnostics in dead birds was done by RT-PCR of brain tissues; if brain tissue was unavailable, diagnostics were occasionally performed on alternative tissues.

In the central region of the Netherlands, mosquitoes were trapped using CO_2 baited CDC miniature light traps (John. W. Hock Company, Gainesville, USA). At intensified sampling locations following WNV detections, CDC Gravid Trap Model 1712 (JW Hock) for collection of gravid females and mosquito aspirators were additionally used.

For nucleic acid extraction from live bird swabs, 600 µL of the viral transport medium (VTM) was used as input material. Tissue samples from dead birds were homogenized in tissue lysis buffer (MagNA Pure DNA Tissue Lysis Buffer) using the Fastprep bead beater (MP Biomedicals, shaking at 4.0 m/s for 10 s). 60 µL of the supernatant was mixed with 540 µL of VTM or Dulbecco's Modified Eagle Medium (DMEM) prior to nucleic acid extraction. Total nucleic acid (NA) was extracted using the MagNA Pure 96 Instrument and MagNA Pure 96 and DNA and Viral NA Large Volume Kit (Roche LifeScience, Basel, Switzerland) according to the manufacturer's instructions. Mosquito pools where homogenized in 1 ml medium (DMEM, $NaHCO_3$, Hepes-buffered saline, penicillin/streptomycin, amphotericin) with one 1/4″ ceramic sphere (MP Biomedicals, Solon OH, USA) using the FastPrep-24 5G bead beater (5 m/s for 30 s). Homogenates were cleared by centrifugation. 60 µl of homogenate supernatant was added to 90 µl MagNA Pure 96 External Lysis Buffer (Roche), and nucleic acids were extracted using Ampure beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA) and a DynaMag 96 side magnet (Invitrogen/Thermo Fisher, Waltham, MA, USA).

Viral whole genome sequencing and phylogenetic analysis

Consensus sequences were generated using the following approaches. Until end 2019, reference based alignment against an arbitrary chosen Usutu virus genome was performed in Geneious[8] or in CLC Genomic Workbench 11.0 (https://www.qiagenbioinformatics.com/), followed by a second reference based alignment to the most closely related sequence to the consensus sequence identified using Blastn[9,10] and generation of a final consensus sequence. From 2020 reference-based alignment against the most similar reference genome from a selected reference set was performed using Minimap2[11]. A consensus genome was extracted, reads were remapped to the generated consensus sequence, and a new consensus sequence was generated. Positions with a coverage below 100 reads and later below 30 reads were replaced with an 'N'. Homopolymeric and primer binding regions were manually checked and resolved by consulting raw reads and reference genomes.

Antibody detection in serum from live free-ranging birds

In the protein micro-array[16], sera were diluted at a 1:80 ratio and tested for IgY reactivity against USUV and WNV NS1 proteins. A signal ≥ 6000 relative fluorescence units (RFU) on the protein micro-array was considered above background. Sera with a signal ≥ 30'000 RFU (near half of the maximum fluorescence value, 67000 RFU and corresponding to an approximate titer of 1:80) were considered highly reactive.

In total, 928 sera with a signal above background on the protein microarray were confirmed using neutralization assays. Initially, Virus Neutralization Tests (VNT) were used. Starting in 2020, Focus Reduction Neutralization Tests (FRNT) replaced VNT as primary confirmation method due to increased sensitivity and specificity. To ensure consistency, all samples previously tested on the VNT with sufficient sample volume remaining were retrospectively re-tested using FRNT. For samples tested on the VNT with insufficient material remaining, the VNT result was retained. Consequently, the final serology results are derived from 785 samples tested with FRNT and 143 samples tested with VNT. Sera were considered positive on the VNT when at least two-serial dilutions showed complete blocking of viral infection (cut-off titer ≥16).

Sera were considered positive on the focus reduction neutralization test when titer was ≥1:160 for USUV and ≥1:80 for WNV (calculated based on a ≥70% reduction in infected cells counts).

Supplementary Results

Supplementary Note 1: Avian Host Species with Usutu Virus or West Nile Virus Occurrence

In addition to Eurasian Blackbirds, Song Thrushes (*Turdus philomelos)*, Eurasian Jays (*Garrulus glandarius*), House sparrows (*Passer domesticus*) and Blue Tits (*Cyanistes caeruleus*) showed high USUV prevalence in live birds (0.2% to 1.8%), with infections also detected in several dead individuals from these species (ranging from 3 to 7). With the exceptions of Blue Tits, USUV-neutralizing antibodies were also detected in these species, with seroprevalence ranging from 0.9% to 4.5%. USUV prevalence was 0.6% in live Redwings (*Turdus iliacus*) and 0.3% in live Great Tits (*Parus major*), with no infections detected in dead birds from these species. Conversely, 7 Common Wood Pigeons (*Columba palumbus,* order Columbiformes) were found dead with USUV infection, while no infections were detected in the small number of live birds tested (n=61). USUV and WNV detection overlapped in 5 species: Song Trushes, House Sparrows, Great Tits, Common Whitethroats (*Sylvia communis*) and Common Chiffchaffs (*Phylloscopus collybita*). The complete breakdown of number of individuals sampled, viral detections and antibodies detections per bird orders and species is presented in **Supplementary Data 1.**

Supplementary Table 1 | Mosquito species sampled and tested at bird ringing sites and via statutory sampling in central Netherlands

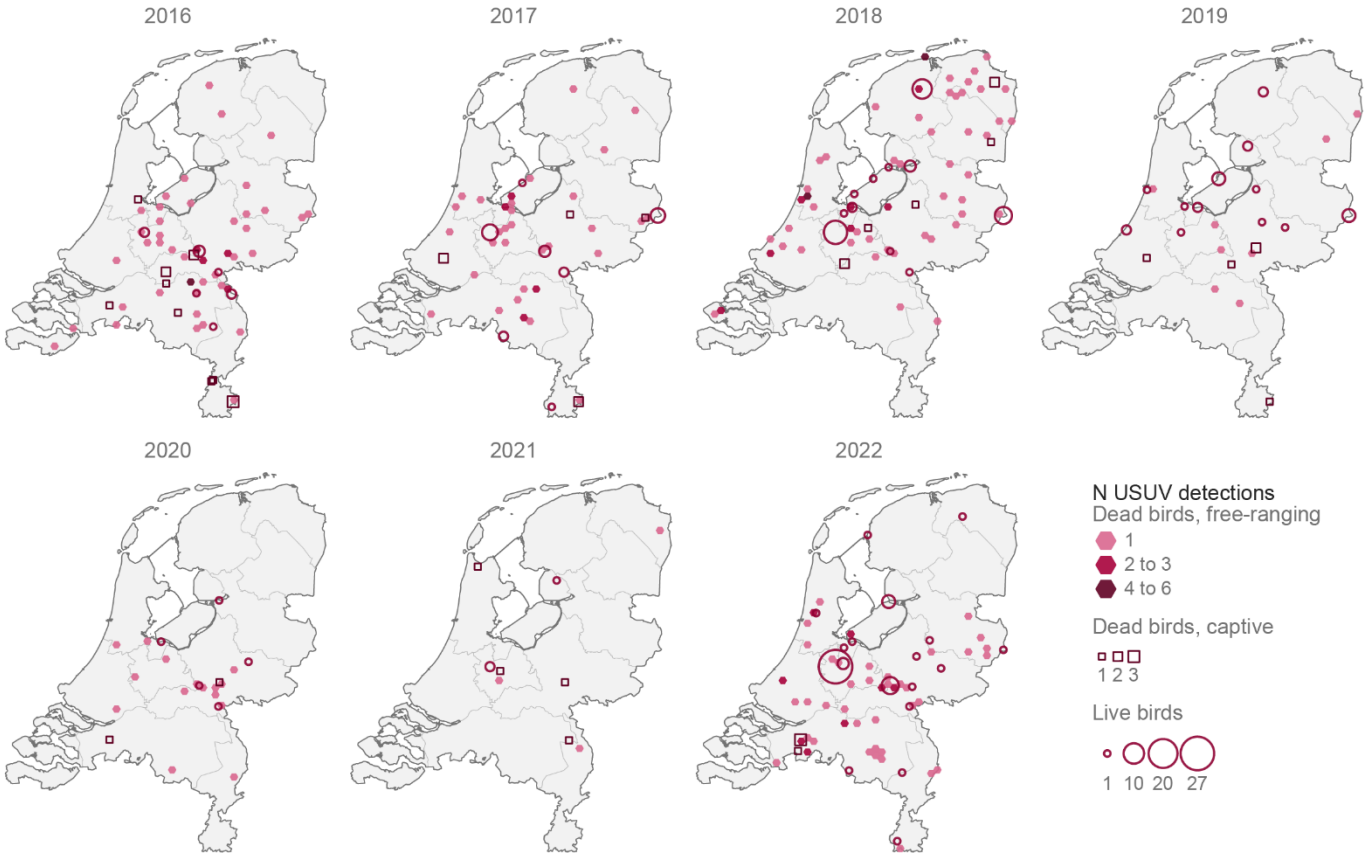
	Species	N pools tested RT-PCR	N individu-als tested RT-PCR	N pools USUV RNA positive	N pools WNV RNA positive	Percent USUV RNA positive pools	Percent WNV RNA positive pools
1	*Culex pipiens/torrentium*	4946	35089	27	6	0.5%	0.1%
2	*Culiseta annulata*	451	1078	0	0	0.0%	0.0%
3	*Anopheles claviger*	233	632	0	0	0.0%	0.0%
4	*Anopheles maculipennis s.l.*	181	257	1	0	0.6%	0.0%
5	*Coquillettidia richiardii*	135	323	0	0	0.0%	0.0%
6	*Aedes cinereus*	109	612	0	0	0.0%	0.0%
7	*Culiseta morsitans*	102	279	0	0	0.0%	0.0%
8	*Culiseta ochroptera*	98	921	0	0	0.0%	0.0%
9	*Anopheles plumbeus*	83	146	0	0	0.0%	0.0%
10	*Aedes vexans*	41	107	0	0	0.0%	0.0%
11	*Culex territans*	23	45	0	0	0.0%	0.0%
12	*Culex sp.*	21	111	0	0	0.0%	0.0%
13	*Aedes sticticus*	12	20	0	0	0.0%	0.0%
14	*Aedes cantans*	10	17	0	0	0.0%	0.0%
15	*Aedes rusticus*	9	9	0	0	0.0%	0.0%
16	*Aedes annulipes*	8	10	0	0	0.0%	0.0%
17	*Aedes sp.*	5	7	0	0	0.0%	0.0%
18	*Culex modestus*	5	15	0	0	0.0%	0.0%
19	*Anopheles sp.*	2	4	0	0	0.0%	0.0%
20	*Culiseta sp.*	2	11	0	0	0.0%	0.0%
21	*Aedes communis*	1	1	0	0	0.0%	0.0%
22	*Aedes detritus*	1	1	0	0	0.0%	0.0%
23	*Aedes punctor*	1	3	0	0	0.0%	0.0%
24	*Aedes rossicus*	1	1	0	0	0.0%	0.0%
	Total	**6480**	**39699**	**28**	**6**	**0.4%**	**0.1%**

Supplementary Table 2 | Number of samples tested and molecular and serological detections of Usutu virus and West Nile virus by research project and year.

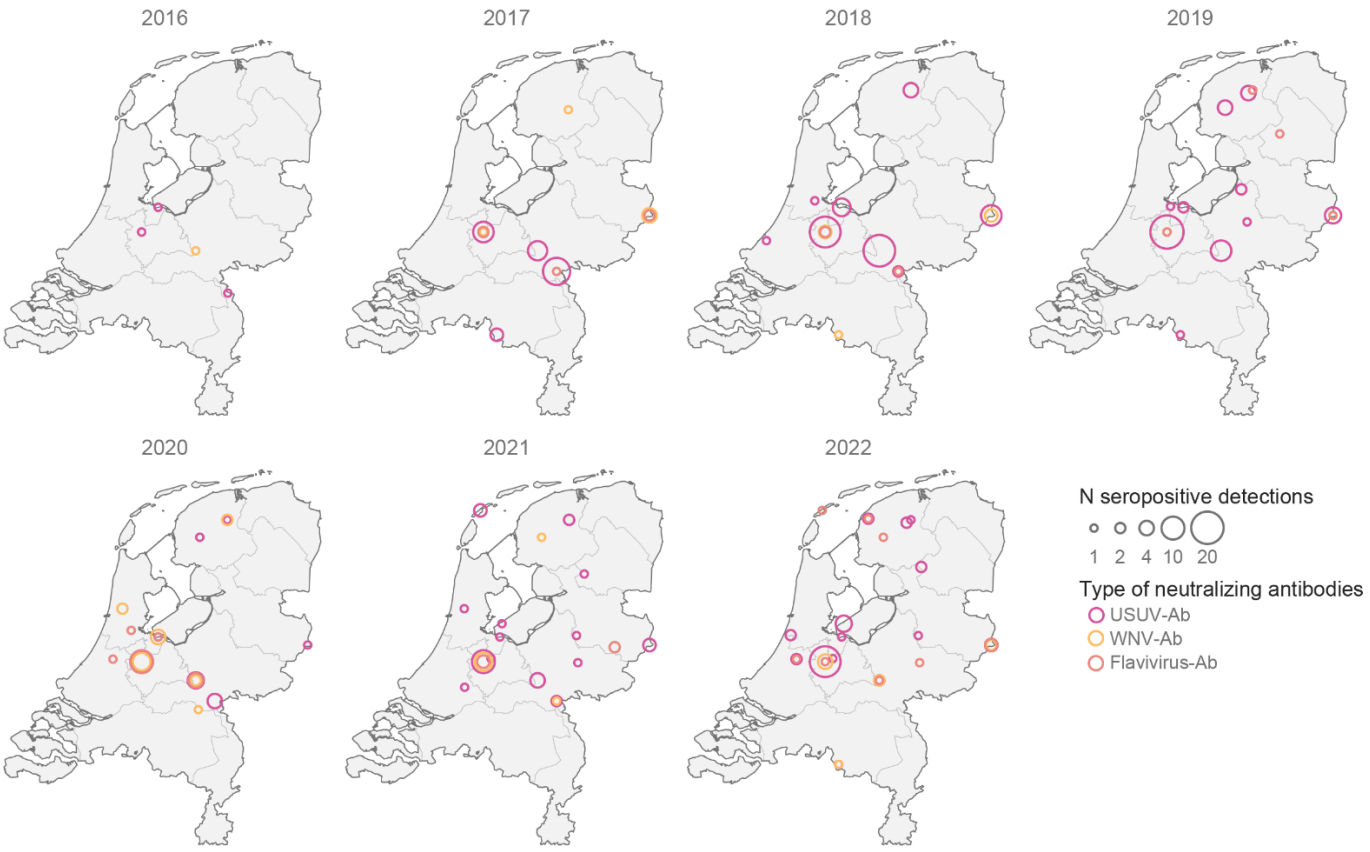
	2016	2017	2018	2019	2020	2021	2022	Total
Live birds - landbirds								
N tested USUV and WNV PCR	627	975	1574	2203	5022	6151	6148	22700
N USUV RNA positive	12	21	39	22	5	2	55	156
N WNV RNA positive	0	0	0	0	8	0	0	8
Percent USUV RNA positive	1.9%	2.2%	2.5%	1.0%	0.1%	0.0%	0.9%	0.7%
Percent WNV RNA positive	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.0%
N tested USUV and WNV PMA	94	320	464	417	657	786	1438	4176
N USUV antibodies positive	3	35	59	51	20	31	41	240
N WNV antibodies positive	1	6	9	2	18	8	9	53
N *Orthoflavivirus*-neutralizing antibodies positive	0	5	3	4	15	5	8	40
Percent USUV-neutralizing antibodies positive*	3.2%	10.9%	12.7%	12.2%	3.0%	3.9%	2.9%	5.7%
Percent WNV-neutralizing antibodies positive*	1.1%	1.9%	1.9%	0.5%	2.7%	1.0%	0.6%	1.3%
Percent *Orthoflavivirus*-neutralizing antibodies antibodies positive*	0.0%	1.6%	0.6%	1.0%	2.3%	0.6%	0.6%	1.0%
Live birds - waterbirds								
N tested USUV and WNV PCR	27	38	192	286	618	3577	5980	10718
N USUV RNA positive	0	0	1	0	0	1	1	3
N WNV RNA positive	0	0	0	0	0	0	1	1
Percent USUV RNA positive	0.0%	0.0%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%
Percent WNV RNA positive	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
N tested USUV and WNV PMA				19	71	27	26	143
N USUV antibodies positive				0	0	1	2	3
N WNV antibodies positive				0	0	0	0	0
N *Orthoflavivirus*-neutralizing antibodies positive				0	2	0	2	4
Percent USUV-neutralizing antibodies positive*				0.0%	0.0%	3.7%	7.7%	2.1%
Percent WNV-neutralizing antibodies positive*				0.0%	0.0%	0.0%	0.0%	0.0%
Percent *Orthoflavivirus*-neutralizing antibodies antibodies positive*				0.0%	2.8%	0.0%	7.7%	2.8%
Dead birds - free-ranging								
N tested USUV PCR	94	110	119	95	230	243	289	1180
N tested WNV PCR	5	18	79	89	230	243	289	953
N USUV RNA positive	53	38	72	8	15	3	54	243
Percent USUV RNA positive	56.4%	34.5%	60.5%	8.4%	6.5%	1.2%	18.7%	20.6%
Dead birds - captive								
N tested USUV PCR	19	10	28	112	175	208	101	653
N tested WNV PCR	0	1	23	112	175	208	101	620
N USUV RNA positive	13	6	7	5	2	4	4	41
Percent USUV RNA positive	68.4%	60.0%	25.0%	4.5%	1.1%	1.9%	4.0%	6.3%
Mosquitoes - Central Netherlands								
N pools tested USUV and WNV PCR				619	1824	1349	998	4790
N individuals tested USUV and WNV PCR				3422	8922	8842	6033	27219
N pools USUV RNA positive				5	0	1	12	18
N pools WNV RNA positive				0	1	0	0	1
Percent pools USUV RNA positive				0.8%	0.0%	0.1%	1.2%	0.4%
Percent pools WNV RNA positive				0.0%	0.1%	0.0%	0.0%	0.0%
Mosquitoes - Bird research stations								
N pools tested USUV and WNV PCR					415	809	466	1690
N individuals tested USUV and WNV PCR					3488	5621	3371	12480
N pools USUV RNA positive					1	0	9	10
N pools WNV RNA positive					5	0	0	5
Percent pools USUV RNA positive					0.2%	0.0%	1.9%	0.6%
Percent pools WNV RNA positive					1.2%	0.0%	0.0%	0.3%

PMA = protein microarray

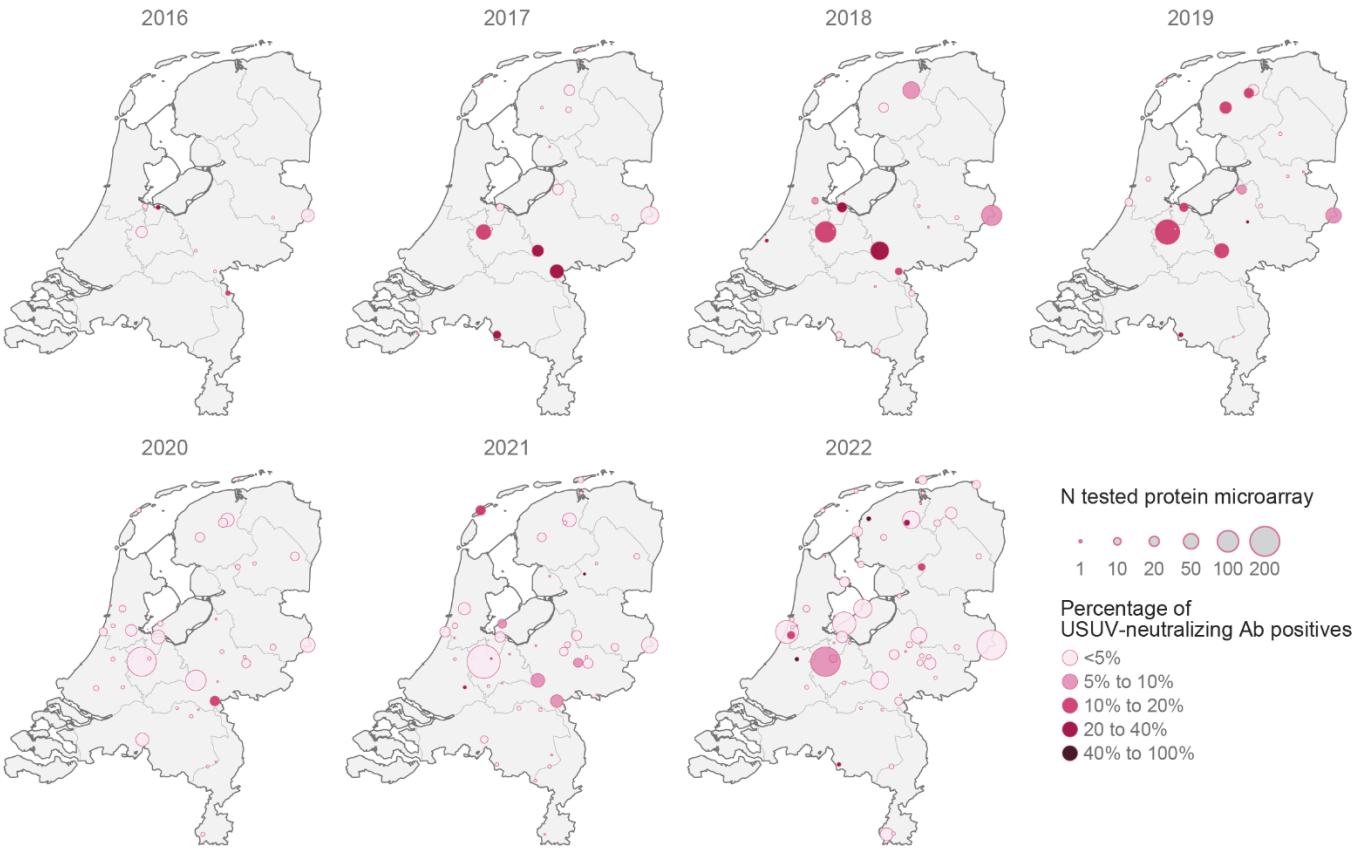
*Percentage of samples tested on the protein microarray that were confirmed positive for neutralizing antibodies by focus reduction neutralization test or virus neutralization test



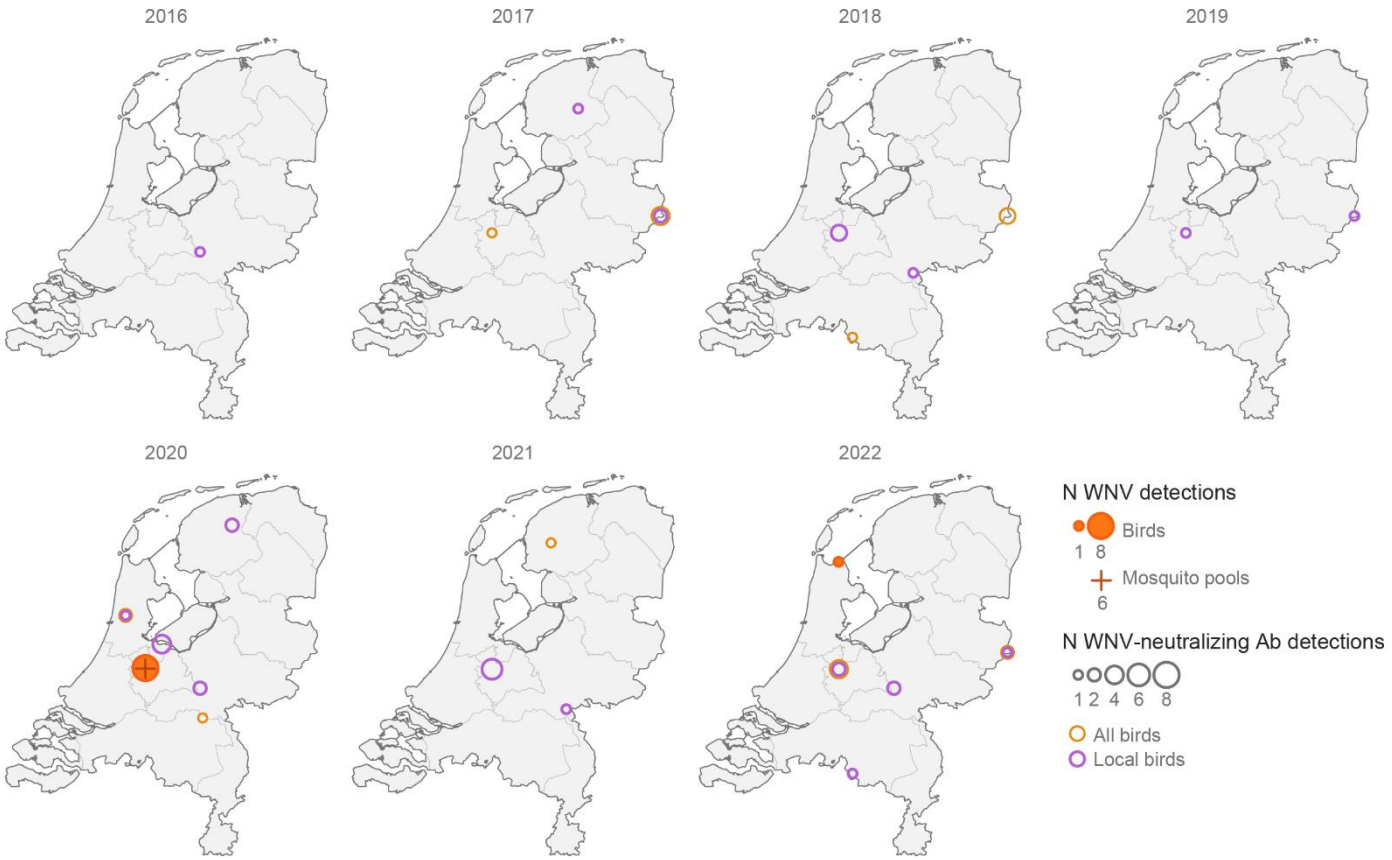
Supplementary Fig. 1 | Geographical distribution of Usutu virus (USUV) detections in birds in the Netherlands per year, 2016-2022. Square symbols indicate detections in dead captive birds, and circular symbols detections in live birds, with sizes proportional to the number of detections. Shading intensity in hexagonal grid cells (size: 30 km²) reflect the number of detections in dead free-ranging birds in that cell. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).



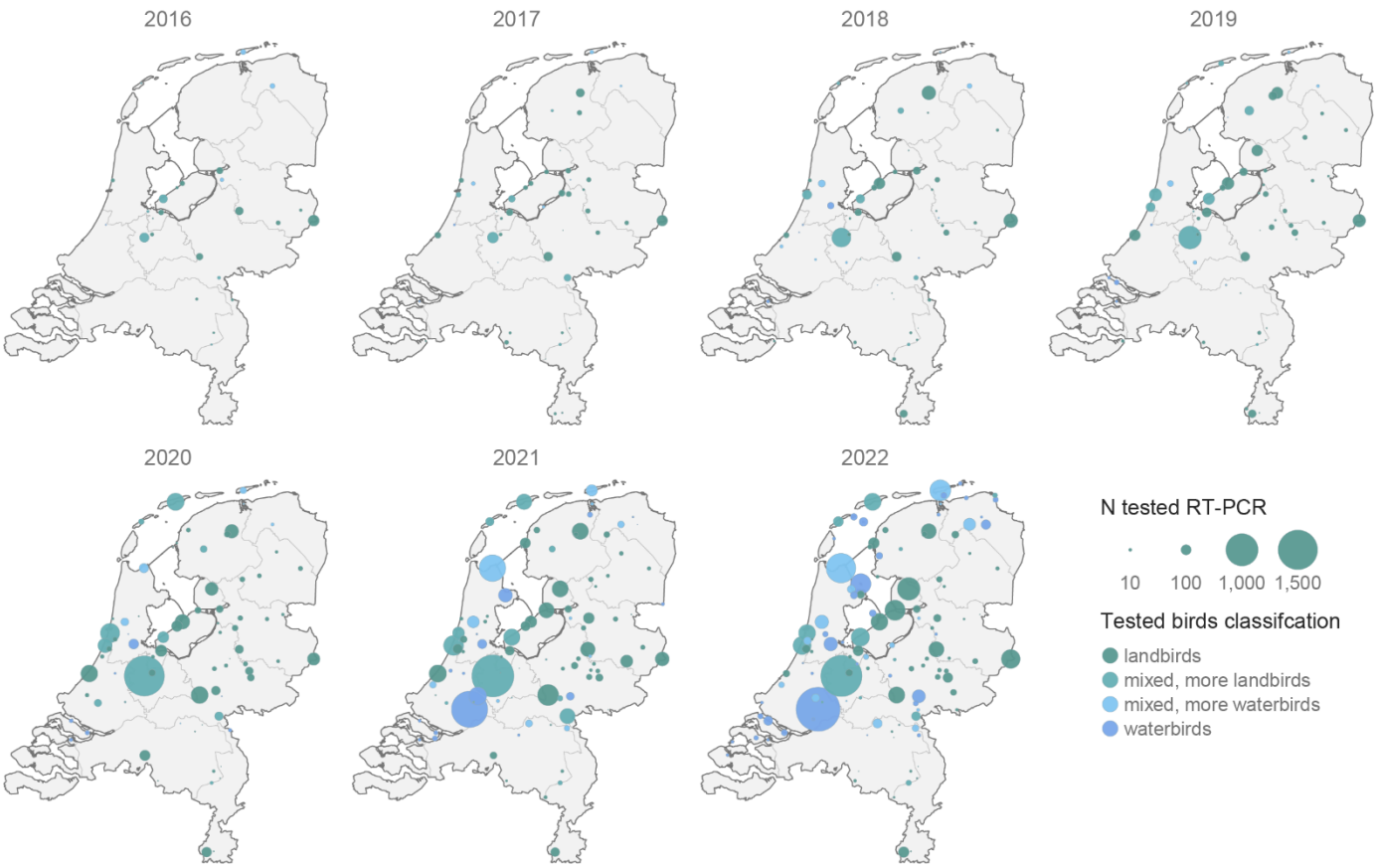
Supplementary Fig. 2 | Geographical distribution of Usutu virus (USUV), West Nile virus (WNV) and *Orthoflaviviruses*-neutralizing antibodies detections in live free-ranging birds per year in the Netherlands, 2016-2022. Circular symbols are coloured per type of antibody and sized proportionally to the number of detections. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).



Supplementary Fig. 3 | Annual distribution of live bird serum sampling and Usutu virus-neutralizing antibody (USUV-neutralizing Ab) seroprevalence by bird ringing area, the Netherlands, 2016-2022. Circular symbols indicate sampling areas from where live bird serum samples were tested for *Orthoflaviviruses* antibodies on the protein microarray, with symbol size corresponding to the number of samples tested. Color intensity indicates the percentage of these samples confirmed positive for USUV-neutralizing Ab by focus reduction neutralization test or virus neutralization test. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).

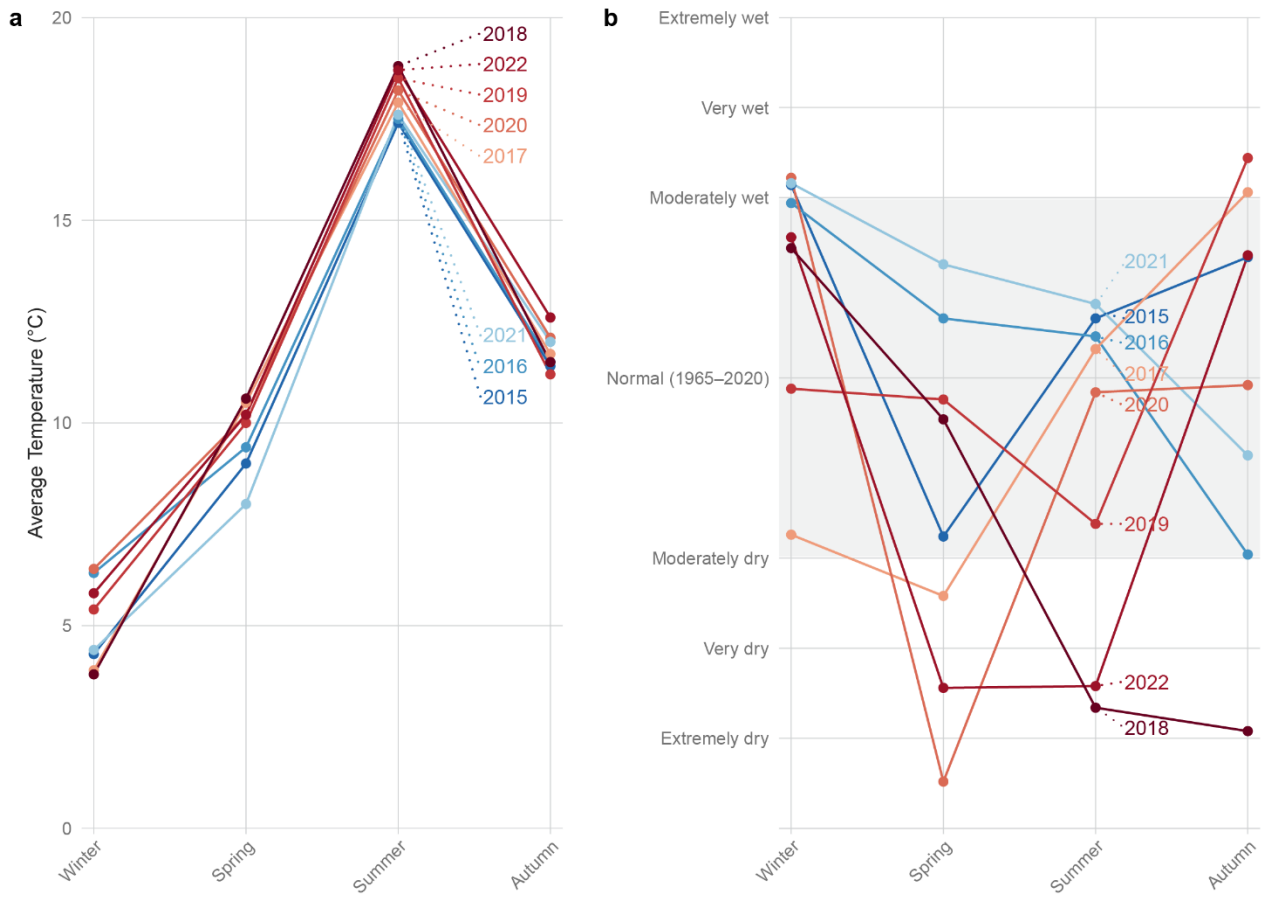


Supplementary Fig. 4 | Geographical distribution of West Nile virus (WNV) and neutralizing antibodies (WNV-neutralizing Ab) detections in live birds and mosquitoes in the Netherlands per year, 2016-2022. Orange symbols indicate molecular detections in live birds (solid circles, sized proportionally to the number of detections) and mosquitoes (cross). Circular outline symbols represent WNV-Ab detections (sized proportionally to the number of detections), with yellow indicating all detections and purple highlighting detections in local birds. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).



Supplementary Fig. 5 | Geographical distribution of live free-ranging birds captures sampled and tested per year in the Netherlands, 2016-2022. Circular symbols are sized proportionally to the number of birds tested per sampling area. The color scheme indicates the predominant bird types sampled. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).

Supplementary Fig. 6 | Geographical distribution of dead free-ranging birds and dead captive birds tested per year in the Netherlands, 2016-2022. Square symbols indicate sites of sampling of dead captive birds, with sizes proportional to the number of birds tested. Shading intensity in hexagonal grid cells (size: 30 km²) reflect the number dead free-ranging birds sampled in that cell. Source administrative boundaries: CBS, Kadaster, "CBSGebiedsindelingen2022" (https://service.pdok.nl/cbs/gebiedsindelingen/atom/v1_0/index.xml).



Supplementary Fig. 7 | Seasonal temperature and dryness/wetness in the Netherlands, 2015–2022. (a) Seasonal average temperatures (°C). **(b)** Dryness/wetness index of seasons per year. This index reflects relative difference between precipitation and potential evapotranspiration (SPEI), compared to a long-term climatological baseline (1965–2020). Values are classified into categories ranging from "extremely dry" to "extremely wet," and provide a standardized measure of meteorological drought and excess moisture. Years are color-coded from blue (cooler) to red (warmer) based on their average summer temperature. Data source: Royal Netherlands Meteorological Institute (KNMI - https://www.knmi.nl/klimaat).

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