

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Dead free-ranging birds cases were reported through the Dutch Wildlife Health Centre (DWHC) online platform (https://dwhc.nl/en/report/form-to-submit-a-dead-animal/). No software was used for meta data collection from other animals. The LightCycler 480 Real-Time PCR System was used for collecting PCR testing results and the ScanArray Express software was used for collecting Protein microarray data.
Data analysis	Custom R scripts were used for data cleaning, epidemiological analysis, and data visualization. The type of code used follows established practices, as described for example in Batra et al. (The Epidemiologist R Handbook, 2021) and Lovelace et al. (Geocomputation with R, 2025). Raw sequence data were analysed using Porechop v0.2.4, fastp v0.20 and Minimap2 v2.17-r941. MUSCLE v3.8.1551 was used for multiple sequence alignment and IQ-TREE v2.0.3 for phylogenetic analyses.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The complete datasets on live and dead free-ranging birds, birds deceased in captivity, and mosquitoes generated in this study have been deposited in the BioStudies database [<http://www.ebi.ac.uk/biostudies>] under accession codes S-BSST1522 [<http://doi.org/10.6019/S-BSST1522>], S-BSST1523 [<http://doi.org/10.6019/S-BSST1523>], S-BSST1505 [<http://doi.org/10.6019/S-BSST1505>], S-BSST1867 [<http://doi.org/10.6019/S-BSST1867>], S-BSST1868 [<http://doi.org/10.6019/S-BSST1868>], S-BSST1875 [<http://doi.org/10.6019/S-BSST1875>] and are also accessible via the Pathogens Portal Netherlands [<https://www.pathogensportal.nl/arboviruses.html>]. Summary tables showing numbers of individuals tested and positive per bird and mosquito species are provided in the supplements (mosquitoes: Supplementary Table 2; birds: Supplementary Data 1). Viral genome sequences and raw reads generated in this study have been deposited on the European Nucleotide Archive (ENA) under project number PRJEB83966 [<https://www.ebi.ac.uk/ena/browser/view/PRJEB83966>].

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

We followed up on five Usutu virus-positive human blood donations collected in the Netherlands in 2018, performing viral genome sequencing analyses. Blood donations were obtained and managed by Sanquin, as described in Zaaijer et al. (Usutu virus infection in Dutch blood donors. Transfusion 2019; 59: 2931–7). Information on the sex and gender of the blood donors was not shared with us.

Reporting on race, ethnicity, or other socially relevant groupings

We do not have information on the race, ethnicity, or other socially relevant groupings of the blood donors, and these aspects were not considered in our study.

Population characteristics

Human blood donations collected in the Netherlands from May to September 2018.

Recruitment

Blood donors were recruited by Sanquin, the national blood supply organization in the Netherlands, through voluntary participation. Recruitment strategies include self-registration, referral by existing donors and public awareness campaigns.

Ethics oversight

USUV sequencing of anonymized USUV-positive human blood donations was approved by the Ethical Advisory Board of the Sanquin Blood Supply Foundation, as described by Zaaijer et al. (DOI: 10.1111/trf.15444).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description

This study investigates the emergence and dynamics of Usutu virus (USUV) and West Nile virus (WNV) in birds and mosquitoes across the Netherlands from 2016 to 2022. Through long-term monitoring, we collected data on free-ranging and captive birds, as well as mosquitoes, to assess viral prevalence, spatio-temporal dynamics, and host range.

Research sample

Live free-ranging birds (N=33'418), sampled to represent free-ranging birds in the Netherlands. Deceased free-ranging birds (N=1'180), collected to represent free-ranging birds with disease-related mortality in the Netherlands. Captive birds that died in zoos or under veterinary care (N=653), sampled to represent the captive bird population in the Netherlands. Mosquitoes (N=39'699), collected to represent the summer mosquito populations across the Netherlands. All were collected from multiple sites across the Netherlands. Various bird and mosquito species present in the Netherlands, as well as captive birds, were sampled; the number of individuals tested per bird and mosquito species are provided in the appendix (mosquitoes: Appendix 1, Table S2; birds: Appendix 2).

Sampling strategy

Live free-ranging birds were captured and sampled by trained volunteer ornithologists across the Netherlands from March 2016 to December 2022. Birds were ringed, weighed and measured. Optimally, throat and cloacal swabs, along with a blood sample and feather samples were collected. Dead free-ranging birds were reported across the Netherlands through a citizen science system from March 2016 to December 2022. Dead captive birds were submitted by zoos and veterinarians. Dead birds were necropsied, and

samples were collected. Mosquitoes were trapped weekly at bird ringing sites throughout the Netherlands between July and October 2020–2022 using fermenting molasses-baited traps. In addition, weekly trapping was carried out in the central Netherlands between May and October 2019–2022, using CO₂ baited light traps. The sample size was determined by specimen availability through these surveillance efforts, and all samples collected during the study period were included.

Data collection

Location, date of capture (for live birds and mosquitoes) or date of death/finding (for dead birds), species, and ring number (for live birds) were recorded for each sampled animal. Metadata for dead free-ranging birds were collected through the Dutch Wildlife Health Centre (DWHC) online platform (DWHC Reporting Form). Live bird metadata were recorded by volunteer bird ringers on paper forms and later transcribed into a digital dataset at the Dutch Centre for Avian Migration and Demography. Mosquito trapping locations and timing were fixed and recurrent, with catch counts per trapping event recorded digitally at the Centre for Monitoring of Vectors of the Netherlands and the Laboratory of Entomology, Wageningen University and Research. Results of molecular and serological testing for live birds and mosquitoes were recorded in digital datasets at Erasmus Medical Center and linked to host metadata. Molecular testing results for one mosquito study were maintained in a digital dataset at the Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM).

Timing and spatial scale

Live free-ranging birds: March 2016 to December 2022 across the Netherlands. Sampling locations were selected to achieve broad geographic coverage but were influenced by the distribution of existing bird ringing stations. Over 200 bird ringing stations are active and operate on a voluntary basis, the frequency and periodicity of sampling vary by station, with weekly sampling year-round at major ringing sites and incidental sampling at other locations. Dead free-ranging birds: March 2016 to December 2022 across the Netherlands; reported by citizens, no set frequency or periodicity. Dead captive birds: August 2016 to December 2022 across the Netherlands, reported by zoo/owner upon deaths, no set frequency or periodicity. Mosquitoes at bird ringing sites: July to October 2020–2022, at bird ringing sites throughout the Netherlands, weekly trapping. Mosquitoes, Central Netherlands: May to October 2019–2022, central Netherlands, weekly trapping.

Data exclusions

No data were excluded from the analyses

Reproducibility

Bird sampling and mosquito trapping followed standardized field protocols to ensure consistency across locations and years. Molecular and serological diagnostics were performed using validated protocols and quality-controlled reagents. Negative and positive controls were systematically included in PCR and serological assays, along with internal controls for PCR testing. Diagnostics were typically performed once per sample, with controls confirming the validity and accuracy of the results.

Randomization

Samples were not allocated into predefined experimental groups. Instead, all samples were collected through passive surveillance, citizen science contributions, and standardized field sampling efforts. As this study was observational, with samples tested as they became available through the sampling efforts, no experimental grouping or randomization was applied.

Blinding

Blinding was not relevant to this study because it was an observational study rather than a controlled experiment. Additionally, molecular and serological diagnostics followed standardized protocols with automated or objective analytical processes, minimizing observer bias.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions

Live birds were captured under various climatic conditions of the European Netherlands, which has a temperate oceanic climate characterized by mild winters and cool summers. The timing of sampling and climatic constraints varied depending on the target bird species. Mosquitoes were trapped during the summer months, which in the Netherlands are temperate and moderately humid, with an average temperature of around 17°C.

Location

Sampling was conducted at multiple locations across the European Netherlands. This region lies between latitudes 50°N and 54°N and longitudes 3°E and 8°E, with elevation ranging from –7 meters to 322.7 meters. The specific locations at which bird and mosquitoes were sampled, along with their coordinates, are provided in the supplementary data of this study.

Access & import/export

Sampling of live free-ranging birds was conducted under ethical permits AVD801002015342 and AVD80100202114410 issued to NIOO-KNAW. The Dutch Wildlife Health Center has an exemption for possession, transport or offering of protected wildlife species in the Netherlands, in order to be able to accept wildlife cadavers for research purposes. No ethical permit is needed for trapping mosquitoes in the Netherlands.

Disturbance

The capture of live birds causes temporary, light to medium distress due to physical restraint, handling, and sample collection. Live bird sampling followed strict ethical guidelines, with trained ornithologists handling birds efficiently to reduce stress. Birds were ringed, measured, and sampled in a manner designed to minimize handling time, and all individuals were promptly released at the capture site.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Mouse Usutu Virus (USUV-NS1) Antibody (MyBioSource, MBS569354), Mouse anti-WNV NS1 antibody (The Native Antigen Company, MAB12160-100)
Validation	No validation statements are provided on the manufacturers' websites. These antibodies were validated for our specific application in de Bellegarde de Saint Lary et al. (Assessing West Nile virus (WNV) and Usutu virus (USUV) exposure in bird ringers in the Netherlands: a high-risk group for WNV and USUV infection? One Health 2023; 16. DOI:10.1016/j.onehlt.2023.100533) and Streng et al. (Sentinel chicken surveillance reveals previously undetected circulation of West Nile virus in the Netherlands. Emerg Microbes Infect 2024; 13: 2406278)

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	Vero cells (ATCC CCL-81)
Authentication	Not authenticated
Mycoplasma contamination	The cell line tested negative for Mycoplasma contamination
Commonly misidentified lines (See ICLAC register)	Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	The study did not involve laboratory animals
Wild animals	Live free-ranging birds in the Netherlands were captured, ringed, measured, and sampled in the field by trained ornithologists. All individuals were promptly released at the capture site. Deceased free-ranging birds in the Netherlands were collected for necropsy and sampling, which were performed at the Dutch Wildlife Health Center. Mosquitoes were trapped using fermenting molasses-baited traps and CO ₂ -baited light traps. Collected mosquitoes were euthanized by storage at -20°C. Euthanizing the mosquitoes was necessary for the screening approach used to detect viral presence in these animals. Various bird and mosquito species present in the Netherlands were sampled. The number of individuals tested per species is provided in the appendix (mosquitoes: Appendix 1, Table S2; birds: Appendix 2).
Reporting on sex	CO ₂ and fermenting molasses-baited traps primarily attract female mosquitoes, as they are seeking a blood meal. Since the goal of this study was to assess virus circulation (where female mosquitoes are the vectors), rather than vertical transmission or other sex-dependent factors, focusing on female mosquitoes was appropriate. The sex of birds was recorded when possible at the Dutch Wildlife Health Center and Vogeltrekstation. However, sex was not a focus of this study, which aimed primarily to determine when and where infections occurred as well as in which species.
Field-collected samples	No laboratory work was conducted on live animals collected in the field. Laboratory work was performed on samples obtained from wild animals under appropriate biosafety and diagnostic laboratory conditions.
Ethics oversight	Sampling of live free-ranging birds was conducted under ethical permits AVD801002015342 and AVD80100202114410 issued to NIOO-KNAW. The Dutch Wildlife Health Center has an exemption for possession, transport or offering of protected wildlife species in the Netherlands, in order to be able to accept wildlife cadavers for research purposes. No ethical permit is needed for trapping mosquitoes in the Netherlands.

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Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.