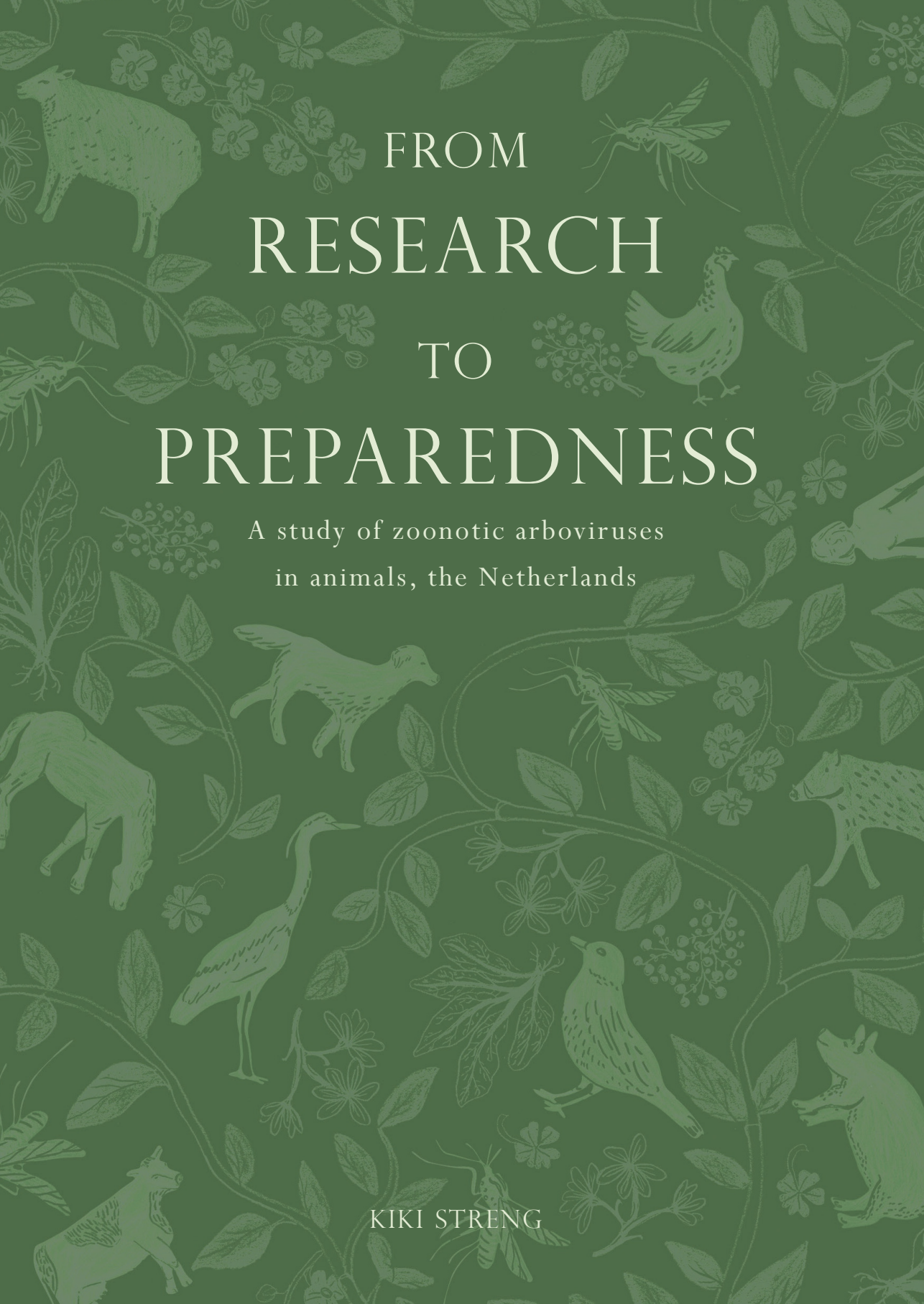


FROM RESEARCH TO PREPAREDNESS

A study of zoonotic arboviruses in animals, the Netherlands

KIKI STRENG

Propositions

1. The perfect sentinel animal for zoonotic mosquito-borne virus surveillance does not exist.
   (this thesis)

2. Veterinary practitioners and the Dutch government have conflicting interests in West Nile virus surveillance.
   (this thesis)

3. Collaboration slows down research.

4. Scientists with an interdisciplinary skillset are undervalued.

5. The word *model* is the most ambiguous word in science.

6. The Dutch healthcare system is a lottery.

7. Individuals without sufficient financial resources should not be allowed to own pets.

Propositions belonging to the thesis, entitled

From research to preparedness: A study of zoonotic arboviruses in animals, the Netherlands

Kiki Streng

Wageningen, 17 December, 2024

From research to preparedness:
A study of zoonotic arboviruses in animals, the Netherlands

Kiki Streng

Thesis committee

Promotors
Prof. Dr Wim H.M. van der Poel
Special Professor, Emerging and Zoonotic Viruses
Wageningen University & Research

Prof. Dr Marion G.C. Koopmans
Department Head Viroscience
Erasmus MC, Rotterdam

Co-promotor
Dr Reina S. Sikkema
Assistant Professor, One Health Virology
Erasmus MC, Rotterdam

Other members
Prof. Dr Gorben Pijlman, Wageningen University & Research
Prof. Dr Gerdien van Schaik, Utrecht University
Prof. Dr Martin Groschup, Friedrich Loeffler Institute, Greifswald - Insel Riems, Germany
Dr Mariet Feltkamp, Leiden University Medical Center and Sanquin Blood Supply

This thesis was conducted under the auspices of the graduate school Wageningen Institute of Animal Sciences.

From research to preparedness:
A study of zoonotic arboviruses in animals, the Netherlands

Kiki Streng

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr C. Kroeze,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 17 December 2024
at 3.30 p.m. in the Omnia Auditorium

Kiki Streng
From research to preparedness: A study of zoonotic arboviruses in animals, the Netherlands
236 pages

PhD thesis, Wageningen University & Research, Wageningen, the Netherlands (2024)
With references, with summaries in English and Dutch

DOI https://doi.org/10.18174/676149

Table of contents

Chapter 1.

General introduction

"Finally, on account of various reasons which it would be idle to relate, I asked myself if it was not the mosquito that transmitted the yellow fever poison." – Dr Charles Finlay, Havana, 1881.

After summing up existing evidence and results from his own experiments, Finlay came to the conclusion that mosquitoes were able to transmit diseases by blood-feeding (1). It was Jesse William Lazear, who proved in 1900 that lab-bred mosquitoes were able to transmit yellow fever in Havana. He died at age 34, after deliberately allowing an infected mosquito to bite him for his own experiment (2). One hundred years later, a plethora of knowledge has been generated about mosquito-borne viruses (MBVs) worldwide. This thesis aims to add a small piece to the MBV puzzle. Results of this thesis are part of a large research consortium of multiple universities and institutes (One Health Predicting Arbovirus Climate Tipping Points, OHPACT). This consortium aims to gain systemic understanding of mosquito-borne diseases, and of how their emergence and transmission is influenced by major environmental and social changes (3). One Health is defined[1] as an integrated, unifying approach that aims to sustainably balance and optimise the health of people, animals, and ecosystems. The recognition that the health of humans, livestock and ecosystems are tightly linked and therefore need to be studied and protected jointly is therefore the starting point of the OHPACT consortium.

1.1 Mosquito-borne viruses

Arboviruses (*ar*thropod-*bo*rne) are viruses transmitted by vectors, such as mosquitoes and ticks (Text box 1). Since the discovery of yellow fever virus (YFV) transmission by mosquitoes in 1900, many arboviruses and their corresponding diseases have been identified. Over 500 arboviruses are currently known (4). Some of the diseases caused by arboviruses have been classified as emerging infectious diseases (EIDs). EIDs can be described as diseases with a significant impact on animal or public health, whose incidence increased over the past decades or is predicted to increase in the foreseeable future (5). These can be divided into two main categories: (i) known pathogens appearing in new host populations or in new geographical areas and (ii) newly identified pathogens. A large proportion of emerging pathogens can be transmitted from an animal reservoir to the human population, so-called spill-over transmission (6). Spill-over, as well as other transmission pathways may lead to outbreaks in the human or animal population. Thereby, outbreaks of (emerging) arboviral diseases can cause substantial impact on human health, animal health, the environment and the economy.

[1] European Union, One Health High Level Expert Panel https://health.ec.europa.eu/one-health/overview_en

Widely known examples of mosquito-borne diseases (Text box 1) in humans are yellow fever, Zika and dengue. Outbreaks of these diseases may cause significant public health impact. For example, about half the world's population is currently at risk of dengue (7,8). An average of 9221 people per year died from dengue between 1990-2013, especially children under 9 years old (9). In 2023, the highest historical record of over 4.1 million new infections were reported. In the first half of 2024, an increase of 231% in cumulative incidence compared to the same period in 2023 was observed (10). Mosquito-borne viruses do not only affect humans. A proportion of MBVs are zoonotic (Text box 1) and thus may also, or only, affect animal health. West Nile virus (WNV), Japanese Encephalitis virus (JEV) and Rift Valley Fever virus (RVFV) are examples of viruses that cause disease in wildlife as well as domestic animals (11). Many zoonotic MBVs belong to the genera *Alphavirus* (family *Togaviridae*), *Orthoflavivirus* (family *Flaviviridae*) and *Phlebovirus* (family *Phenuiviridae*). Examples of viruses from these three genera are Sindbis virus (SINV), WNV and RVFV, respectively (12,13).

Text box 1 – Definitions

In this thesis, I refer to **animals** as domestic and wild animals, not being humans or insects/vectors

Arboviruses are viruses that are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous (blood-sucking) arthropods.

Vector-borne diseases (VBDs) are diseases caused by pathogens transmitted by vectors, including midges, ticks and sandflies. In contrast to arboviruses, these also include parasitic and bacterial pathogens. Examples are bluetongue, Lyme and leishmaniasis.

Mosquito-borne viruses (MBVs) are per definition arboviruses as they are transmitted by mosquitoes.

Mosquito-borne diseases are diseases caused by pathogens that are transmitted by mosquitoes. This also includes diseases such as Lyme or malaria (caused by bacteria or parasites).

Zoonotic diseases, or zoonoses, are diseases shared between animals – including livestock, wildlife, and pets – and people. Zoonotic diseases can be foodborne, waterborne, or vector-borne, or transmitted through direct contact with animals, or indirectly by fomites or environmental contamination. (14)

Next to public and animal health impact, outbreaks of MBVs can lead to major economic impact. Estimating the economic impact caused by mosquito-borne diseases poses significant challenges within scientific research. This is because the impact encompasses not only the direct costs associated with illness, surveillance, control and prevention efforts but also indirect factors such as the decline in tourism (7,14). This was exemplified by the Zika outbreak of 2016, with an estimated cost of

US$ 18 billion in Latin America and the Caribbean (15). Socio-ethical impacts of MBV outbreaks are linked with public and animal health impact, as outbreaks can lead to decreased welfare. Societal issues may arise due to culling of animals, reduced welfare of pets and reduction in recreational spaces (16). Production losses, deaths and culling of animals affect food security and the livelihoods of humans. A striking example is Rift Valley fever (RVF), the disease caused by RVFV. Infections of this virus in ruminants cause high mortality and abortion storms in Africa and the Arabian Peninsula (17). The Somali Region in Ethiopia, a livestock dependent economy, experienced a 35% reduction in GDP ($135 million) due to trading bans implemented to control the spread of RVF (18). Moreover, MBVs also have an environmental impact encompassing the loss of affected species and effects of vector control by insecticides used to control or prevent transmission (19,20). This loss of species and impacts of vector control have the potential to disrupt ecosystems. However, the exact cascading effects are not yet fully understood (20). Therefore, quantifying the environmental impact of MBVs remains difficult (16).

The potential impact of MBVs on either humans, animals or both, depends largely on their transmission cycle (Figure 1) as viruses can be transmitted between different vector and host species. Transmission requires a competent vector and host, in which amplification of the virus occurs (13,21). The majority of MBV vectors are from the *Culex, Aedes,* and to a lesser extent *Anopheles* and *Culiseta* spp. *Culex* mosquitoes are present worldwide and serve as the main vectors for many MBVs such as WNV, USUV, SINV and RVFV (22). *Aedes* mosquitoes, especially *Ae. aegypti* and *Ae. albopictus*, are known as the main vectors for YFV, dengue virus (DENV) and Zika virus (ZIKV). *Aedes aegypti* is widespread in the Americas, Africa, Asia and, to a lesser extent, Oceania (23). *Aedes albopictus* is even more widespread as it is also established in (southern) Europe and is rapidly expanding its range (23,24). The transmission cycle of DENV and ZIKV does not require an animal host. Such a transmission cycle is referred to as an urban (epidemic) cycle (25). For viruses with an enzootic cycle, wild animals such as birds serve as amplifying hosts. In addition to that, some viruses achieve (further) amplification in domestic animals; the epizootic cycle (21). JEV is a virus with an enzootic and epizootic cycle as it amplifies in (wild) birds as well as in domestic pigs (26). Spill-over to dead-end hosts may occur from enzootic as well as epizootic cycles (Figure 1). Dead-end hosts are hosts that can get infected, but in which the virus does not replicate to high enough levels to infect a vector and thus does not contribute further to the transmission of the virus. Infection mainly occurs via a mosquito bite, but for some viruses such as WNV, infection of humans may occur via blood or organ donation from infected donors (27,28). Examples of dead-end hosts are horses (*Equus caballus*) and humans in the case of WNV (29,30). This thesis mainly focuses on zoonotic MBVs with an enzootic and/or epizootic transmission cycle (Figure 1).

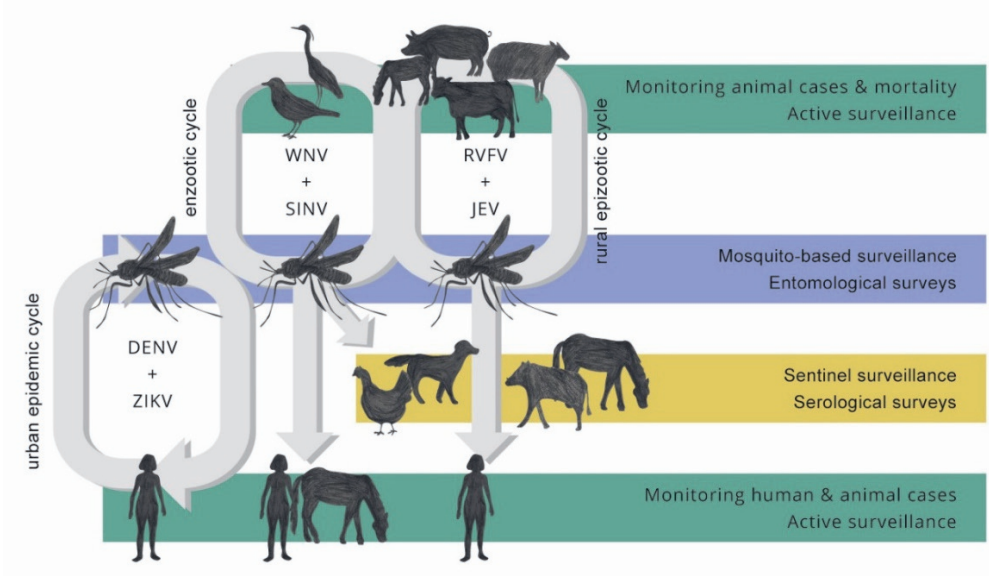


Figure 1. Mosquito-borne virus (MBV) transmission cycles and surveillance options (for definitions, see Text box 2). Example viruses: dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), Sindbis virus (SINV), Rift Valley Fever virus (RVFV) and Japanese Encephalitis virus (JEV). Urban (epidemic) cycle: amplification in humans and mosquitoes (no animal reservoir needed). Enzootic cycle: amplification in wild animals and mosquitoes. Epizootic cycle: amplification in domestic animals and mosquitoes. (Figure adapted from (25))

1.2 Drivers of emergence

Emergence of MBVs is instigated by an array of factors, also referred to as drivers. Major drivers of MBV emergence are (1) climate & weather, (2) travel & trade and (3) land use & farming practices (31,32). Abundance and migration patterns of (reservoir) hosts such as wild birds are impacted by climate change (33). Additionally, climate change has a large effect on local MBV emergence as vector presence, abundance and competence are largely dependent on temperature and humidity (34,35). Travel and trade have an impact on the spread of MBVs over large as well as short distances (36). Infectious vectors or hosts may be introduced by trade and humans travel more frequently and in larger numbers, increasing introduction risk (37,38). For instance, the introduction of invasive exotic mosquito species such as the Asian tiger mosquito (*Ae. albopictus*) into new regions was due to trade of used tyres (39,40). As the transmission of viruses like DENV and ZIKV is dependent on these exotic vectors, transmission only occurs if vectors are established (23).

Land use changes can be grouped in changes in water management, deforestation, agricultural development and urbanization (41,42). Effects of land use change on MBV transmission are not yet fully understood. Suggested effects are mainly linked to host

and vector community diversity and abundance and their responses to anthropogenic changes, co-infection, pathogen communities and pathogen-host-vector interaction (35,41). A notable illustration of the interplay between land use and farming practices is observed in the case of JEV transmission. The interplay of ardeids (Ardeidae), (feral and domestic) pigs and mosquito vectors is closely linked with landscape and climate. Ardeids are reservoirs for JEV and their habitat consists of (grassy) wetlands, lakes, rice fields and floodplains. The main mosquito vector (*Culex* species mosquitoes) requires stagnant water bodies. In Australia, water accumulated at specific land use types due to La Niña phases in 2022. Transient wetlands, waterways, cultivated land and fragmented grasslands were strongly associated with JEV outbreaks in piggeries during that year (43). This vector-animal-human interface is particularly relevant as it favours spill-over to humans (44).

In an era of global change, we may expect to see an increase in circulation of MBVs in Europe, as well as their spread into new geographical regions due to increasing (global) travel, trade and migration (45). Furthermore, Europe is the fastest-warming continent in the world (46). Multiple strategies and policies have been designed and implemented for climate adaptation to reach climate neutrality (47). The common agricultural policy (CAP) was adopted by the European Union (EU) in 2021 which will result in changes predominantly in agricultural and rural areas (48). Climate change does not necessarily (only) lead to an elevated risk of MBVs. For instance, rising temperatures and droughts might actually reduce the risk of MBV circulation in some regions (49). Forecasting the emergence and spread is thus challenged by the involvement of numerous factors and drivers. Therefore, early detection of vectors, viruses and infections in their (potential) hosts is crucial to prevent the consequences of disease.

1.3 Emergence of mosquito-borne viruses in Europe

In Europe, multiple MBVs have emerged over the past decades, including Usutu virus (USUV), WNV and SINV. Since the temperate European climate is characterized by seasonal changes in temperature, the warm summers allow for mosquito-borne transmission of these viruses (50). Introduction of MBVs with a bird reservoir, such as WNV, is thought to be facilitated mainly by migratory birds (16,51,52). Multiple introductions of USUV and WNV to Europe have occurred from the 1950s onwards (51,53). The first USUV epidemic was observed in Austria in 2001 (54). After their establishment in Europe, both USUV and WNV expanded northwards in the years after. In 2016, a large USUV outbreak occurred in the Netherlands, leading to massive die-off in blackbirds (*Turdus merula*) and owls (Strigiformes) (55). The virus has been detected in the Netherlands every year since (56). Since 2004, after the introduction of WNV lineage 2 to Hungary, an explosive spread of WNV and an increase in human cases was observed in Europe. This was particularly evident in 2018, when a massive outbreak led

to over 2,000 human and 285 equine cases across Europe (29). In 2020, the virus was first detected in the Netherlands and human cases were identified in two regions (57,58). Interestingly, a different pattern was observed for SINV, an alphavirus with a wild bird reservoir. A recent study by Ling et al. (59) suggests that only a single introduction from central Africa to Sweden in the 1920s was needed for establishment. The virus first became endemic in Sweden, after which it was introduced to Finland in the 1960s and southward into Germany in the 1970s. This southward spread, which is in contrast with what was observed for USUV and WNV, is suggested to be due to autumn migration of the main reservoir bird species.

1.4 Mosquito-borne virus surveillance

Given the increasing emergence of MBVs, surveillance (Text box 2) is imperative to enable swift responses in terms of public health policies, control measures and prevention strategies. As vaccines and antiviral drugs remain unavailable for most MBVs (60), it is essential to detect introduction, spread and new potential (reservoir) hosts of MBVs rapidly. These detections allow for prevention and control of spread in the form of, for example, communication campaigns to increase awareness and reduce mosquito numbers (61). The capacity of a large proportion of MBVs to amplify within wildlife species constrains the ability to manage their emergence and spread. Nonetheless, active control of mosquitoes, travel or trade restrictions and blood and organ donor screening may be initiated after infections have been detected (62,63).

Text box 2 – Definitions

Surveillance: the systematic ongoing collection, collation, and analysis of information related to animal health and the timely dissemination of information so that action can be taken. There are six main mechanisms of surveillance: 1. Voluntary notification, 2. Mandatory notification, 3. Outbreak investigation, 4. Sentinel surveillance, 5. Structured surveys, 6. Censuses (5,68)

Passive surveillance: the examination of only clinically affected cases of specified diseases (continuous monitoring of the existing disease status of the populations that are studied, using routinely collected data which includes statutory notification of disease to produce outputs that can feed into policy decisions) (68)

Active surveillance: sampling of clinically-normal animals in the population (important with subclinical cases and carriers) (68,69)

Monitoring: the intermittent performance and analysis of routine measurements and observations, aimed at detecting changes in the environment or health status of a population (5)

Furthermore, data generated by surveillance, such as virus detections or case notifications, can in turn be used in statistical or mathematical models to identify risk factors, create risk maps or even attempt to predict future outbreaks. Output from

these models can contribute to change or refinement of the surveillance or response system (64,65).

Options for MBV surveillance consist of human, animal, vector (Figure 1) and environmental surveillance. The complex transmission cycles of MBVs necessitate comprehensive surveillance, as focusing solely on one aspect of the transmission cycle is insufficient to acquire a complete understanding of the ecology and epidemiology (25,66). Methods for detection of circulation are mainly focused on observation of disease or mortality, detection of the virus or detection of antibodies against the virus of interest. Observations of disease and mortality can only be obtained if the virus of interest causes significant morbidity (and mortality). Isolation of the virus itself provides evidence for circulation in space and time, and is possible in vectors, reservoir and dead-end hosts. Another benefit of virus detection is genetic characterisation of the pathogen, which provides essential insight in epidemiological patterns as well as adaptations that alter interactions with vectors and/or hosts (56,59,67). However, in dead-end hosts, rapid viral clearance often results in a narrow time window for virus detection. Antibodies generated after infection can often be detected in reservoir and dead-end hosts, regardless of whether they have shown clinical signs. Detection of antibodies provides insight into past infections, but lacks information on the exact time and location of infection (25). Furthermore, cross-reactive antibody binding of viruses or viral antigens from the same genus may be observed (68). Notable examples are viruses from the genera *Orthoflavivirus* (family *Flaviviridae*) and *Alphavirus* (family *Togaviridae*). Cross-reactive antibody binding of viruses within specific serogroups can hamper accurate identification of past infections. Finally, vaccinations may elicit long-lasting neutralizing antibody responses, which makes it more difficult to confirm infections. Monitoring disease and mortality in humans and animals relies on notification of (suspected) cases to the appropriate authorities. This in turn relies on awareness of physicians and/or veterinarians to detect possible cases (25). An interesting example is the case of SINV in Scandinavia. Phylogenetic analyses of SINV sequences by Ling et al. (59) indicate that SINV was introduced into Sweden in the 1920s, but the first case in Sweden was only detected in 1967. This suggests that the virus was circulating undetected for many decades before it was reported to cause disease. In Finland, SINV has been a notifiable disease since 1995, and numbers of reported cases are a 10-fold higher than in Sweden, where awareness is considerably lower. It is yet to be determined whether this difference can be fully attributed to difference in awareness, or that other factors may play a role (59). Other options of surveillance in humans are cross-sectional or repeated surveys for virus detection or serology. These surveys may be set up in specific risk areas, for example in outbreak investigations. Advantages of sampling humans compared to animals are the ability to obtain more accurate information on

travel history and vaccination status, high sample volumes and opportunities for follow-up sampling. Furthermore, testing of human blood samples is often carried out in regions where MBVs have been detected to prevent infections through donor organs or blood products. Information obtained from this screening may also be used in surveillance programmes.

Mosquito-based surveillance is mainly focused on monitoring of competent vector presence and abundance. Additionally, mosquitoes may be tested for specific pathogens or blood-fed mosquitoes may be analysed to assess their feeding behaviour or presence of pathogens in their bloodmeals (25). According to the definition of the World Organisation for Animal Health (WOAH), mosquito presence and abundance data fall under environmental surveillance, but I list it separately here. Environmental surveillance from weather and climate data do not provide proof of circulation but may assist in surveillance, given that transmission cycles are heavily influenced by weather conditions, as previously mentioned (69). Temperature and rainfall data can inform models that help to identify high risk areas for targeted surveillance. Wastewater, feathers and eDNA are other examples that may provide useful sources of environmental data (70–72).

Multiple European countries, such as France and Italy, have already implemented comprehensive surveillance systems for WNV and USUV that encompass several of these surveillance components (73,74). Surveillance of MBVs in the Netherlands is further discussed in section 1.6.

1.5 Role of animals

Animals can play a significant role in MBV transmission, especially in enzootic and epizootic transmission cycles. Infections can be detected in reservoir hosts, but also in dead-end hosts after spill-over has occurred (25). According to the European Animal Health Law (AHL), surveillance to observe abnormal fatalities or symptoms of listed diseases is mandatory. Whether a disease is listed is based on: (1) the disease profile (morbidity, mortality etc.), (2) impact of the disease, (3) potential to generate crisis situation or use in bioterrorism, (4) feasibility, availability and effectiveness of disease prevention and control measures and (5) the impact of disease prevention and control measures. The pathogen of interest is assessed by an expert committee using a standardized method (75). A brief description of the listing categories (A-E) and mosquito-borne disease examples are shown in Table 1. Infections caused by JEV, RVFV and WNV are listed as category E diseases. The early detection and notification of these diseases requires a certain level of awareness and monitoring/surveillance. Early detection of RVF (category A) is even more eminent as control and eradication from the EU is mandatory according to the AHL (Regulation 2016/429 on transmissible

animal diseases). Category A diseases also require contingency plans and outbreak simulations to evaluate these plans.

In addition to legal obligations, surveillance in animals offers several advantages. Clinical cases and mortality in animals may precede detection in humans. Well-known examples are the observation of clinical WNV infections or mortality in crows (*Corvus brachyrhynchos*) or horses before human cases appear (76–78). As spill-over of MBVs is dependent on a plethora of factors such as vector and host abundance, vector host-preference and land use, the ability to detect infections of MBVs in dead-end hosts varies per region (69,79). In areas with low numbers of horses, for example, it is possible that human WNV cases are not preceded by horses, as was observed France in 2018 (80). The observation and notification of animal cases as an early detection method is also called passive (symptomatic) surveillance (Text box 2).

Table 1. Categories of listed diseases in the Animal Health Law (AHL) of the European Union (EU). Rift Valley fever (RVF), Venezuelan Equine Encephalitis (VEE), West Nile fever (WNF), Japanese Encephalitis (JE). Check marks indicate surveillance/control/eradication is mandatory for this category, crosses indicate this aspect is not mandatory. *No mosquito-borne disease listed in this category.

	Short definition	Surveillance	Control	Eradication	Example
A	Does not normally occur in EU and immediate eradication measures must be taken as soon as it is detected	✓	✓	✓	RVF
B	Must be controlled in all Member States with the goal of eradicating it throughout EU	✓	✓	✓	*
C	Measures are needed to prevent it from spreading to parts of the EU that are officially disease-free or that have an eradication program	✓	✓	Optional	*
D	Measures are needed to prevent it from spreading on account of its entry into the EU or movements between Member States	✓	✓	✗	VEE, RVF
E	Disease for which there is a need for surveillance within the EU	✓	✗	✗	WNF, JE, RVF, VEE

In contrast to passive surveillance, active surveillance (Text box 2) involves sampling of clinically normal animals in a population (81). Active surveillance offers the advantage of not requiring to wait for cases to emerge and provides the option to target surveillance efforts to specific areas. This is especially relevant when asymptomatic infections are common (low morbidity) or to obtain or remain a disease-free status. Furthermore, it allows for observing trends in specific populations or areas that may be at increased risk. Examples of active surveillance are seroprevalence studies or serosurveys (25,66). Seroprevalence studies provide information on the proportion of individuals in a population with antibodies in their blood, indicating they have been exposed to the virus of interest. These studies can aid in assessing risk factors and indicate potential risk areas. Additionally, they provide information on the proportion of animals that is still susceptible to infection.

Animals can be used as sentinels for MBV occurrence and spread. The word sentinel originates from the Latin word “sentire”, which means “to feel” or “to perceive”. In English, sentinel originally referred to a guard or lookout who was tasked with keeping watch and alerting others to potential threats or dangers. A classic example of a sentinel animal is the "canary in the coal mine” used to detect carbon monoxide. The collapse of a canary served as an early-warning for toxic gases (82). The canary sentinel fits the definition by Stahl (1997): "*any non-human organism that can react to…an environmental contaminant before the contaminant impacts humans.*" 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”* (5). This definition thus includes animals that develop clinical signs after infection, as well as asymptomatic animals that develop antibodies (84). Sentinels may be reservoir or dead-end hosts. Sentinel surveillance can be used to detect a pathogen in a new area, changes in the prevalence or incidence of a pathogen over time, the extend (and direction) of spread, the range of infected hosts, or the effect of intervention strategies (85). Notably, the canary in the coal mine is not regularly tested and does not classify as a sentinel animal according to this definition.

Very few studies that mention the term “sentinel” provide a definition for the term, and if they do, they often refer to different definitions. An important aspect in WOAH’s definition is the notion of regular testing, which implies repeated testing of the same individual(s). Often, animals are suggested as MBV sentinels but have only been tested in cross-sectional (sero)surveys. For some these species regular testing could be feasible, while for other (often wildlife) species, repeated sampling is often impossible.

Examples of sentinel animal species for MBVs in studies that meet the WOAH definition:

- Chickens (*Gallus gallus domesticus*) (86–89)
- Pheasants (*Phasianus colchicus*) (90)
- Various zoo animals (91–93)

For some species, both cross-sectional serosurveys and repeated sampling studies are reported and indicate these might be suitable as sentinels:

- Dogs (*Canis lupus familiaris*) (94–98)
- Domestic pigs (*Sus domesticus*) (99–101)

Often, cross-sectional studies mention the potential use of specific species as sentinels. In these cases, animals were not repeatedly sampled:

- Many wild bird species (102–105)
- Equines: Horses (95,96,106,107), donkeys (*Equus asinus*) and mules (*Eq. asinus* × *Eq. caballus*) (108)
- Wild boars (*Sus scrofa*) (100,109)
- Cattle (*Bos taurus*) (101)
- White-tailed der (*Odocoileus virginianus*) (100,110)

Which animal species is suitable for the proposed surveillance system is dependent on the purpose of the system (85). For MBVs, the objectives of the system are often linked to rapid detection of outbreaks, evaluation of control programmes, identification of new and emerging diseases or proving the absence of a specific disease (81). In addition, a suitable sentinel species is dependent on the pathogen of interest, the population at risk (often humans) and the sentinel population. It is important to consider the species' response to infection, relationship with the population of concern and the transmission cycle (85), and whether the main vectors of the virus of interest feed on the animal species.

1.6 Rationale of this thesis

The Netherlands is particularly vulnerable to outbreaks of viral diseases transmitted by mosquitoes due to its water-dominated landscape, dense human, livestock and companion animal population (111,112). In 2010, this was already acknowledged in the Dutch Emerging Zoonoses Information and Priority system (EZIPs) (113). The EZIPs list was updated in 2019, and JEV (3rd), WNV (9th) and RVFV (13th) are examples of MBVs listed, out of a total of 86 zoonotic pathogens. The 2010 report mentioned the following recommendations (among others):

- *"Zoonotic vector-borne infections, emerging for the Netherlands, should receive focused, structured and structural attention."*
- *"Start or strengthen zoonotic (syndrome) surveillance systems for arthropods, wildlife, exotics and companion animals (pets and horses). Project-based studies to estimate the presence or prevalence of zoonotic pathogens in these animal populations should first be performed."*
- *"State-of-the-art models, focusing on specific aspects and questions rather than trying to be all-encompassing, are needed, for example, approaches that incorporate mathematical/ mechanistic models with statistical models based on trap data and high- (e.g., land use data) and low-resolution (e.g., climate data) satellite information."*

Currently, the surveillance of MBVs in animals in the Netherlands consists of: mandatory notification of cases (as mentioned in 1.5), pathology and testing of dead wildlife (114), and serological testing of animals for export. In recent years, project-based surveillance in mosquitoes and wild birds (USUV, WNV and SINV) and horses with neurological symptoms (WNV) was set-up. These projects were both led by research consortia such as One Health PACT, as well as government institutes. After the first detections of WNV in 2020, a surveillance and response plan was developed based on input from multiple institutions involved in the WNV response team (115). The action plan mentions the importance of ecological surveillance to assess if there are specific risk areas for virus circulation, to get an indication of the infection risks for humans and horses. Furthermore, the importance of animal surveillance as early-warning method is mentioned in light of the delay of detection for human cases, which can be a few weeks or even longer (115). The action plan ran from 2021-2023 and in 2024 most surveillance initiatives have halted. Next to the early detection of MBVs, it is important to get insight in the current seroprevalence in animals, to assess which animal species would serve as useful sentinels, it is important to perform seroprevalence studies in multiple species, including animals with asymptomatic infections. These studies can also reveal historical infections, providing insight in potential risk areas.

1.7 Aim and outline of this thesis

The overall objective of this thesis is to provide insight in the epidemiological situation, (response) surveillance options and potential risk of MBVs in animals in the Netherlands, in order to inform future research and surveillance strategies. The chapters of this thesis are focused on three main themes: knowledge about the presence of MBVs in the Netherlands, options for animal surveillance in response to virus detections and preparedness for future outbreaks. The individual chapters cover (research on) identified knowledge gaps with regards to (sero)prevalence of MBVs,

suitability of animal species for MBV surveillance, options for response surveillance and future transmission risk of MBVs in the Netherlands. An outline of the chapters and how they relate to these overarching themes is given in Figure 2. The details of prevention, management and control of outbreaks (e.g., the development and effect of control measures such as vaccines or communication campaigns) will not be addressed in this thesis.

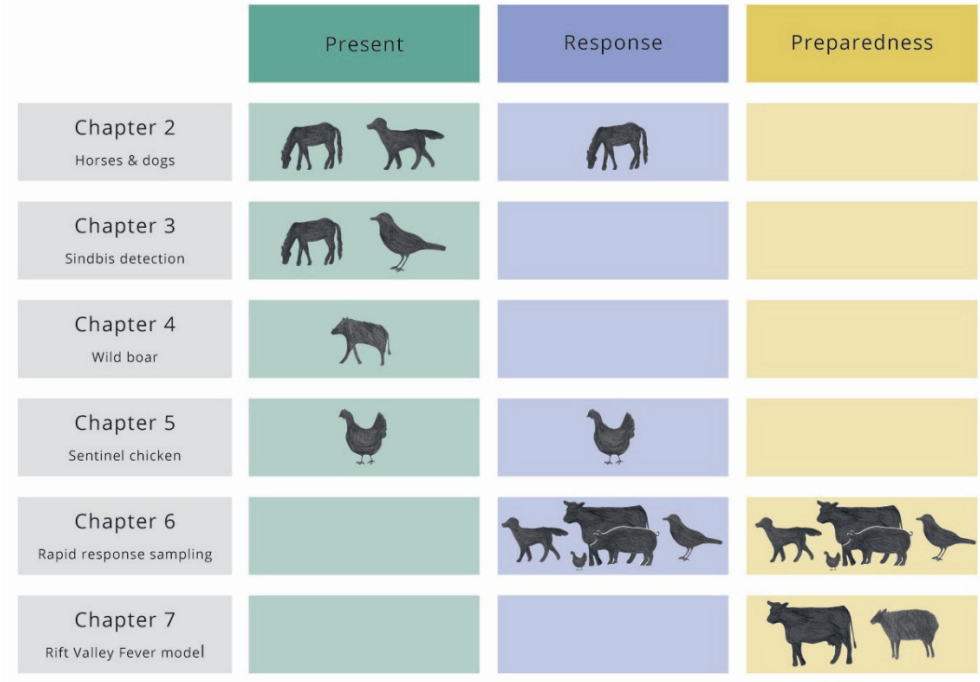


Figure 2. Schematic overview of the chapters of this thesis and their relation to the three main themes. Icons illustrate which animal species are addressed in the chapter.

The (sero)prevalence of MBVs in the Netherlands is largely unknown. Therefore, in **chapter 2** we investigated the baseline seroprevalence of orthoflaviviruses in horses and dogs in the Netherlands. A serosurvey was conducted over a timespan of a year, in which horse and dog sera were collected and tested for neutralizing antibodies against orthoflaviviruses. Secondly, we assessed the knowledge and Health Belief of Dutch horse owners towards MBVs, with an emphasis on WNV. Passive surveillance of WNV is dependent on awareness and knowledge of horse owners, as they are the first in line to observe symptoms in their animals. Furthermore, their intention to use preventive measures for themselves or their horse may aid in prevention of WNV spill-over.

Currently, there is no evidence of SINV presence in the Netherlands. The virus has already been present in Sweden and Finland for decades, and more recently evidence of circulation was detected in Germany. To investigate whether SINV reached the

Netherlands, we decided to test the horse samples collected in chapter 2 for neutralizing antibodies against SINV in **chapter 3**. Next to horses, we tested humans, mosquitoes and wild birds sampled during the same time period for the presence of SINV RNA and/or neutralizing antibodies.

To assess the potential use of wild boars in MBV surveillance, **chapter 4** focuses on this species. Wild boars are present mainly in the (south)east of the Netherlands, and samples are routinely collected to maintain a disease-free status for African Swine Fever (ASF) and other diseases. We selected serum samples from a four-year period and tested those for neutralizing orthoflavivirus antibodies. The results were further analysed using a 3-level Bayesian hierarchical model to elucidate cross-reactive and co-circulation patterns of USUV and WNV.

After the first detection of WNV in the Netherlands in 2020, research initiatives were instigated to assess the (continued) circulation and spread of WNV and USUV. In **chapter 5**, a serological study in the two regions where WNV cases were identified is described. With this study, we aimed to assess whether petting zoo and backyard chickens could serve as suitable sentinels for WNV and USUV in the Netherlands. This aim was divided in two topics: (1) assess the seroprevalence of USUV and WNV in the outbreak regions and (2) study the persistence of antibodies against USUV and WNV in chickens. In order to do this, we repeatedly sampled chickens in both regions over a time period of one year. Additionally, we collected mosquitoes at some of the chicken sampling locations. Serum samples of chickens were tested for USUV and WNV neutralizing antibodies and blood-meal analyses were performed to identify the animal species mosquitoes had fed on.

After detection of (newly) emerged viruses, a rapid response is necessary. This response encompasses not only the communication and control measures, but also the investigation of spread of the virus by the use of additional surveillance activities. Animal samples may aid in the rapid identification of new areas of circulation or unidentified (reservoir) hosts. Possibly, this could be done with the use of existing animal samples. However, an overview of all sample collection and storage initiatives is currently not available in the Netherlands. Therefore, in **chapter 6**, we identified existing animal sample streams through a literature search and interviews. Through these interviews, we also investigated the barriers and conditions related to use of these existing samples. Furthermore, additional interviews with government institutes and legal experts were performed to assess the options for active surveillance in case use of existing samples is not possible.

This future outlook is essential as the Netherlands is a country with an extremely high human and animal density. As mentioned in literature and recommended in the EZIPs report, statistical or mathematical models can be used to identify risk areas or predict

the impact of outbreaks of MBVs that have not emerged yet. **Chapter 7** describes a model that was used to try to estimate outbreak potential of RVFV in the Netherlands. The aim of this study was to compare the current (reference) situation with to two distinct hypothetical future scenarios. Recognizing that climate change and policy decisions will significantly shape the country's future, we aimed to assess the effect of land use and climate change, combined with changes in agricultural policies on potential RVFV outbreaks.

In **chapter 8** I will summarize and discuss the main findings, conclusions and limitations of this thesis. This discussion will be focused on MBV circulation and possibilities for research and surveillance with a focus on the Netherlands in different animal species. Furthermore, I will discuss the use and interpretation of serological methods, implementing One Health arbovirus research and surveillance in practice, and raise ethical questions related to MBV surveillance in animals. Finally, I will share recommendations and focus for future MBV research.

Acknowledgements

I would like to thank Wim van der Poel, Marion Koopmans, Reina Sikkema and Rody Blom for providing useful feedback to earlier versions of this chapter.

Chapter 2.

Orthoflavivirus surveillance in the Netherlands: Insights from a serosurvey in horses & dogs and a questionnaire among horse owners

Kiki Streng[1], Renate W. Hakze-van der Honing[2], Heather Graham[2], Sophie van Oort[2], Pauline A. de Best[3,4], Ayat Abourashed[3,5], Wim H. M. van der Poel[1,2]

Published in Zoonoses and Public Health (2024)

Affiliations
[1] Quantitative Veterinary Epidemiology, Wageningen University and Research, Wageningen, the Netherlands
[2] Wageningen Bioveterinary Research, Wageningen University and Research, Lelystad, the Netherlands
[3] Viroscience, Erasmus MC, Rotterdam, the Netherlands
[4] National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.
[5] Centre for Advances Studies of Blanes, Blanes, Spain

Abstract

Zoonotic arboviruses (*ar*thropod-*bo*rne) of the *Orthoflavivirus* genus, such as West Nile virus (WNV), Usutu virus (USUV) and Tick-borne encephalitis virus (TBEV), are emerging in Northwestern Europe and pose a threat to both human and animal health. In the Netherlands, passive symptomatic surveillance (notification of clinical cases) in horses is one of the main pillars for the early detection of WNV. For such passive surveillance to work properly, horse owners and veterinarians need to recognize symptoms and report suspected cases to the authorities. Currently, little is known about the seroprevalence of orthoflaviviruses in domestic animals in the Netherlands. Therefore, this study aims at identifying the seroprevalence of WNV and USUV in horses and dogs in the Netherlands. Additionally, this study seeks to evaluate the knowledge and perceptions of Dutch horse owners towards mosquito-borne viruses.

A cross-sectional serosurvey in horses and dogs was conducted between May 2021 and May 2022. Serum samples were screened using an ELISA and doubtful and positive samples were confirmed by Virus Neutralization Tests for WNV, USUV and TBEV. A validated questionnaire, the MosquitoWise survey, was used to assess the knowledge and perceptions of Dutch horse owners towards mosquito-borne viruses between July and October 2022. The serosurvey revealed a low seroprevalence for WNV in horses and no WNV positive dogs were found. Similarly, a low USUV seroprevalence was found in dogs. The MosquitoWise survey revealed a high knowledge level for horse owners and high awareness of WNV vaccination but a more limited intent to vaccinate.

The low seroprevalences of WNV and USUV indicate many dogs and horses remain susceptible, offering opportunities for trend analysis and surveillance. However, despite multiple recent detections of WNV, USUV, and TBEV in humans, the role of dogs and horses in early detection of human cases is debatable. High awareness among horse owners and the absence of detected equine WNV cases highlight this uncertainty. Continued surveillance is crucial for detecting increased virus circulation and protecting both animal and human health.

Introduction

Zoonotic arboviruses (*ar*thropod-*bo*rne) of the *Orthoflavivirus* genus are emerging in Northwestern Europe and pose a threat to human and animal health. Three orthoflaviviruses have been detected in the Netherlands so far: Usutu virus (USUV), West Nile virus (WNV) and Tick-borne encephalitis virus (TBEV) (117). The viruses are maintained in a cycle of enzootic circulation between mosquitoes and birds in the case of USUV and WNV and between ticks and mammals in the case of TBEV (117). Spill-over to dead-end hosts can occur through bites of infected vectors. Although infection often goes unnoticed in animals, clinical disease has been reported in birds (USUV and WNV), horses (WNV and sporadically for USUV and TBEV) and dogs (WNV and TBEV) (11). In humans, WNV and TBEV infections generally cause mild, flu-like symptoms but can develop into severe neurological disease in a small percentage of cases. In contrast, USUV infection rarely leads to severe human disease (117).

Mosquito-borne orthoflaviviruses have been present in Europe for over two decades. USUV was first detected in Austria in 2001 and, retrospectively, found in Italy in samples from 1996 (118). WNV is one of the most widespread arboviruses in the world and has been present in Europe since 1996. Both USUV and WNV have now been detected in many parts of Europe. However, no clinical cases have been detected in the United Kingdom (UK) and Scandinavia (62,119). Bagaza virus (BAGV), an orthoflavivirus from the Ntaya serocomplex, appears to be restricted to Spain and Portugal (120). Tick-borne orthoflaviviruses TBEV and Louping ill virus (LIV) have been present in Europe for almost a century. LIV detection is primarily limited to the British Isles, but cases have also been detected in Norway and Spain (121). TBEV is widespread in Europe, including the UK (122). Over the past decade, orthoflaviviruses have been moving towards the Netherlands, resulting in the first detection of USUV in 2016 (123). USUV caused large bird die-offs in the Netherlands during an outbreak and has been detected in birds and mosquitoes every year since its detection (55,56). Since 2016, twelve human cases of TBEV have been identified, and the virus has also been detected in ticks and wildlife (124). In 2020, WNV was first detected in the Netherlands in birds and mosquitoes (58). Since then, eight human cases have been identified in two regions (57,125). The virus was detected again in a Grey Heron (*Ardea cinerea*) in a new region in 2022 (126).

Within the *Orthoflavivirus* genus, there are multiple serocomplexes that consist of antigenically related viruses (68,127). USUV and WNV are part of the Japanese encephalitis (JE) serocomplex, while TBEV and LIV are part of the TBE serocomplex (127,128). Antibodies within a serocomplex may show significant cross-reactive responses in serologic analyses. Moreover, cross-reactions even occur between viruses from the JE and the Ntaya serocomplex (129). Cross-reaction hampers the diagnosis of clinical cases, as well as, the estimation of seroprevalence in a population. As many orthoflaviviruses co-circulate in parts of Europe, co-infection, or sequential infection of multiple viruses in one host, may occur. This further complicates the diagnosis and correct identification of the infecting virus(es). For seroprevalence studies, the general approach is to use a screening method with a sensitive but less specific test (30,117,130). Positive samples are then confirmed using Virus Neutralization Tests (VNTs) or similar neutralization tests (30,130). To conclude which virus had been the infecting virus, titres between cross-reactive viruses are compared and a fourfold difference or greater is regarded to indicate the infecting virus (30).

Early-detection and surveillance strategies are regularly used to protect human and animal health from emerging arboviruses. Usual surveillance strategies include vector, animal and human surveillance or a combination of these (117). In the Netherlands, passive symptomatic surveillance in horses is one of the pillars of early detection of WNV, in addition to human, bird and vector surveillance (115). For the symptomatic surveillance to work accurately, horse owners and veterinarians need to recognize symptoms and report suspected cases to the authorities (116). As mammals do not often display overt clinical signs of disease, serological investigations are often used in surveillance programs to investigate past virus circulation or spread. Commonly used mammal species for serological investigations are horses, dogs and wildlife, such as deer and wild boar (100,131–133). However, horses can be vaccinated against WNV. While vaccination protects against disease, it also interferes with serological diagnostics. This interference is mainly seen for IgG detection, but IgM detection after recent vaccination has also been described (128,134,135). As such, the use of horses for surveillance is limited in areas where a large proportion of the population has been vaccinated (117).

To date, few studies have investigated the seroprevalence of the abovementioned viruses in the Netherlands (136,123,137). However, cross-sectional or longitudinal serosurveys may provide useful information on risk regions and risk factors, which may guide future surveillance or intervention strategies. The Netherlands has a large equine community with an estimated number of 450,000 horses and 400,000 active riders (138). In addition, around 1.8 million dogs are kept as pets (139). Thus, these widely

distributed species may be useful in arboviral surveillance. Additionally, no studies have been performed to investigate perceptions and preventive behaviours of horse owners towards mosquito-borne viruses (MBVs) in the Netherlands. This is crucial because these perceptions and behaviours are key in the current symptomatic surveillance strategy.

In this study, we set out to determine baseline seroprevalences for USUV and WNV in horses (*Equus caballus*) and dogs (*Canis lupus familiaris*). Additionally, we investigated horse owner perceptions and preventive behaviours towards MBVs, with a special focus on WNV, by using an online questionnaire. By combining these two methods, we aimed to get a better understanding of the potential role of domestic animals for orthoflavivirus surveillance in the Netherlands.

Materials and methods

A cross-sectional serosurvey was used to estimate seroprevalences of WNV and USUV in horses and dogs in the Netherlands. Additionally, horse owners and caretakers' (hereafter called horse owners) perceptions and preventive behaviours towards MBVs were assessed by using an adapted version of the MosquitoWise survey (140).

1. Serosurvey

1.1 Study design and sampling

The required sample size was 381 samples per species (CI 95%, error 1%), with an estimated seroprevalence of 1% and an estimated population of 450,000 horses and 1.8 million dogs (141). Veterinary practices were recruited through advertisements in e-mail, digital newsletters, social media (Facebook and LinkedIn), the Netherlands Journal of Veterinary Science and personal communication. All veterinary practices treating horses, dogs or both were eligible to participate in the study.

Animals were selected by the participating veterinarians. Animals were only enrolled if they were over one year old, had never been abroad and were never vaccinated against WNV (due to its interference with the diagnostic tests). Informed consent was acquired from owners prior to including their animal(s) in the study. A maximum of five animals per holding were included. Sampling took place from 1 May 2021 until 1 May 2022.

Serum samples were collected by jugular or cephalic venepuncture. A questionnaire was filled out for each animal (in Dutch, available in supplementary material B). At the laboratory, sera were stored at -20°C until testing.

1.2 Serological assays

Laboratory analyses were performed at the Dutch national reference laboratory for notifiable diseases in animals in Lelystad. All samples were initially screened for orthoflavivirus antibodies by a commercial WNV enzyme-linked immunosorbent assay (ELISA) (ID Screen®, West Nile Competition Multi-species, IDvet Innovative Diagnostics, France). This ELISA detects anti-E protein antibodies of both USUV and WNV and is known to show cross-reactions to other orthoflaviviruses such as TBEV (130). In addition to the standard manufacturer protocol, a dilution series of the positive control was tested (1:8 and 1:16). The signal to noise (S/N%) was calculated by using the mean optical density (OD) value of the two negative controls. Horses sampled from the 1st of May until the 2nd of November 2021 (with sufficient amount of serum left) or with doubtful and positive competition ELISA results were also tested for WNV IgM antibodies by a commercial ELISA (ID Screen®, West Nile IgM Capture, IDvet Innovative Diagnostics, France). This was done to test for recent infections.

Doubtful and positive horse and dog samples were subsequently tested by Virus Neutralization Tests (VNTs). Sera were tested in duplicate in VNTs for WNV, USUV and TBEV, according to a previously described method (30). In short, sera were inactivated for 30 minutes at 56°C. Three-fold serial dilutions were made ranging from 1:10 to 1:2430 for WNV and USUV and from 1:20 to 1:4860 for TBEV. Subsequently, 50ul containing about 100 TCID50 of either WNV (GenBank accession no. HQ537483), USUV (GenBank accession no. OP007489) or TBEV (GenBank accession no. M77799) was added to each well and incubated at 37°C in 5% CO2 for 1.5 hours. The virus titre was confirmed by performing back titrations alongside the VNTs. Positive (experimentally infected horse for WNV, field infected bird for USUV, vaccinated human for TBEV) and negative (Dutch horses pooled sample, 2007) controls were included. Finally, 50uL of virus susceptible cells were added, meaning 15000 cells/well Vero-CCL81 cells for WNV and USUV or 7500 cells/well BHK-21 cells for TBEV. Plates were then incubated for four (TBEV) or six (WNV and USUV) days at 37°C in 5% CO2. Readout was performed by scoring each well for the degree of cytopathic effect (CPE). Titres of each replicate were calculated using the reciprocal dilution at which 50% or more CPE was present. The titre for each sample was calculated as the average of titres of both duplicates after logarithmic transformation. Titres of ≥10 (USUV and WNV) or ≥20 (TBEV) were considered positive as determined by testing reference samples of naturally and experimentally infected equines and/or birds and negative equine samples. Additionally, samples just below ELISA cut-off (S/N% values 50-60) were tested in all VNTs, and none of those had a neutralizing titre. Infection with a specific virus was confirmed if the VNT titre for the specific virus was at least four-fold higher compared to the other virus(es). In case the titre difference was smaller, results were classified as undetermined orthoflavivirus exposure. To double-check if animals with

positive samples complied with all inclusion and exclusion criteria, veterinarians of ELISA-positive animals were contacted and asked to confirm vaccination and travel history.

2. MosquitoWise survey

The MosquitoWise survey was developed for a European-wide audience and is based on an adapted Health Believe Model (HBM) (140). The survey was designed to measure health belief and intent to show preventive behaviour against MBVs. Additionally, other questions to obtain information on demographics and sources of information were included. For this study, seven additional questions were added and are shown in supplemental material C. These questions were shown only to Dutch participants who answered "*Yes*" to the question "*Do you own or take care of at least one horse for a minimum of one day per week?*". Horse owners were recruited through advertorials in newsletters from Dutch equine organisations (KNHS, KWPN and FNRS), social media posts (LinkedIn, Twitter and Facebook) and an online equine forum (Bokt.nl). The recruitment period ran from July 2022 until October 2022, after which the questionnaire was closed.

3. Data analysis

MosquitoWise answers were extracted from the LimeSurvey platform in November 2022. Data from both the seroprevalence study and MosquitoWise survey were stored in Microsoft Excel (version 2208). Postal codes were converted into two-digit postal codes to ensure pseudonymization of owner data. Results were analysed with R by using RStudio, version 2023.03.1+446. The observed seroprevalence was calculated as the percentage of VNT confirmed samples for a specific virus out of the total sample size for that species. MosquitoWise knowledge scores were determined by awarding one point to each correct knowledge question answer and then calculating the total number of points (min. 0, max. 9 points). Participants were then divided into minimum (0-2 points), intermediate (3-6 points) and high knowledge (7-9 points). Reverse barrier scoring was used in calculation of the HBM sum score and in the barriers construct score. A higher barrier construct score, thus, indicates lower barriers for using prevention measures were experienced by respondents.

Results

1. Serosurvey

In total, 61 veterinary practices agreed to participate in the study. Eventually, 47 practices submitted one or more serum samples during the study period. They collected 628 sera: 258 from dogs and 370 from horses (see Table 2). Samples were collected in all 12 provinces of the Netherlands. Most samples originated from Utrecht

(24%), South-Holland (19%) and North-Brabant (19%). All data obtained in the serosurvey is shown in supplemental material A.

Table 1. Results of ELISA positive sera tested in VNTs. (WNV: West Nile virus, USUV: Usutu virus and TBEV: Tick-Borne Encephalitis virus). †age in years

Animal ID	Species	Age†	WNV	USUV	TBEV	Conclusion
4	Horse	14	<10	<10	<20	Negative
67	Horse	13	<10	<10	<20	Negative
118	Horse	5	<10	<10	<20	Negative
122	Horse	10	30	<10	<20	WNV infection
133	Horse	5	<10	<10	<20	Negative
179	Horse	16	5.7	<10	<20	Negative
194	Horse	24	<10	<10	<20	Negative
208	Horse	13	<10	<10	<20	Negative
249	Horse	17	<10	<10	11.5	Negative
258	Dog	10	<10	<10	<20	Negative
328	Horse	18	<10	<10	<20	Negative
343	Dog	6	<10	<10	<20	Negative
366	Horse	11	<10	<10	<20	Negative
447	Dog	7	5.7	<10	<20	Negative
488	Dog	10	10	10	<20	Undetermined orthoflavivirus
525	Dog	7	<10	<10	<20	Negative
593	Dog	14	<10	17.3	<20	USUV infection
602	Horse	12	Cell toxicity		<20	Not interpretable

1.1 Serology

Seven dogs (7/258) and 18 horses (18/370) had doubtful or positive ELISA results. These 18 horses all tested negative for IgM antibodies. All competition ELISA negative horses sampled from 1st of May – 2nd of November 2021 (n=166) also tested negative for IgM antibodies, except for one horse with a borderline doubtful result (S/P 36%). Seven ELISA positive animals (6 horses and 1 dog) were excluded from further analyses. Four horses appeared to have been vaccinated, two had been abroad and there was insufficient serum present to perform VNTs for one dog. More information on the excluded animals can be found in supplemental material D. Table 1 shows the results of all ELISA positive sera tested in VNTs. A spatial overview of the results of the serosurvey is shown in Figure 1. The map shows that the seropositive animals were located near areas where USUV, WNV and TBEV (includes virus and serological

detections) had been detected in mosquitoes, wildlife or humans from 2016 until May 2022 (56,58,142).

Based on these results, an observed seroprevalence of 0.27% (1/364, 95% CI [0.00-0.81]) was found for WNV in horses. One dog was USUV positive, resulting in an observed seroprevalence of 0.39% (1/257, 95% CI [0.00-1.15]).

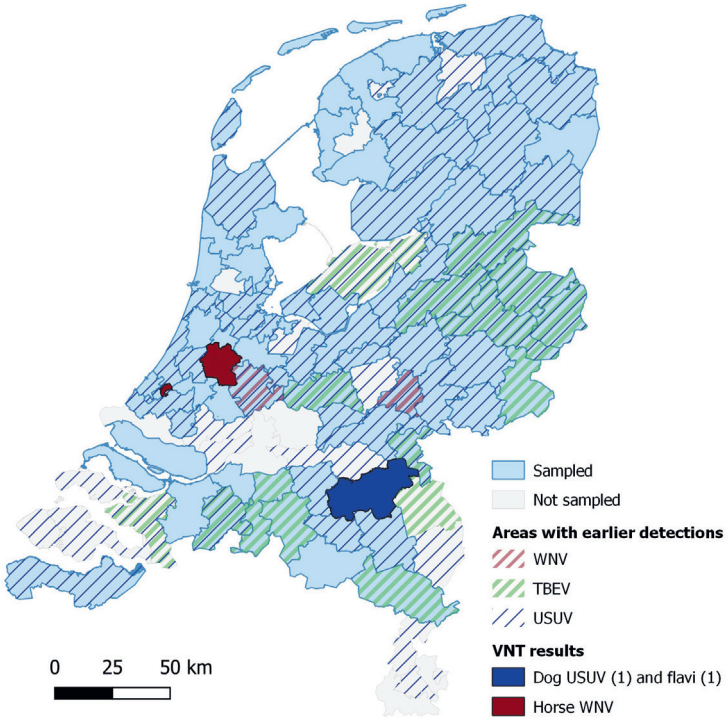


Figure 1. Serosurvey results shown in two-digit postal code areas. Earlier detections (colour shaded areas) published up to the end of the serosurvey in May 2022 are included (56–58,143). Map was created by using QGIS, version 3.22.5.

1.2 Serosurvey questionnaire

Table 2 shows the owner responses to the serosurvey questionnaire. Most dogs (79%) were housed in urban areas, while most horses (75%) were housed in agricultural areas. The amount of time spent outside differed systematically between both species. While most dogs daily spent 0-6 hours outside, the majority of horses spent >6 hours outside. Interestingly, a higher percentage of dogs received insect repellents, yet more dogs than horses had one or more ticks removed during the 12 months before sampling. Due to the small number of detected WNV and USUV infections in this study, we could not perform any further analyses on risk factors based on answers from the owner questionnaire.

Table 2. Results of the serosurvey owner questionnaire, N(%) for horses and dogs separately. †median (Inter Quartile Range)

	Species	
	Horse, N = 364	Dog, N = 258
Age	12 (6, 17)†	4 (1, 8)†
Sex		
Female	196 (54%)	139 (54%)
Male	168 (46%)	119 (46%)
Housing area		
Agricultural	273 (75%)	43 (17%)
Agricultural/nature	8 (2.2%)	2 (0.8%)
Urban	51 (14%)	204 (79%)
Urban/nature	3 (0.8%)	3 (1.2%)
Nature	28 (7.7%)	5 (1.9%)
Amount of time spent outside per day (/24h)		
0-6 hours	92 (25%)	203 (79%)
6-12 hours	139 (38%)	40 (16%)
12-18 hours	30 (8.2%)	7 (2.7%)
>18 hours	103 (28%)	6 (2.3%)
Use of repellent in vector season		
No	236 (65%)	88 (35%)
Yes	128 (35%)	166 (65%)
Tick(s) removed within 12 months prior to sampling		
No	328 (90%)	171 (67%)
Yes	35 (9.6%)	83 (33%)
Type of outdoor space		
Paddock	33 (9.1%)	-
Pasture	154 (42%)	-
Both	176 (48%)	-
Use of a blanket during vector season (April-November)		
No	224 (62%)	-
Yes	140 (38%)	-
Number of horses on premise		
1-2	51 (14%)	-
3-10	141 (39%)	-
10-20	32 (8.8%)	-
>20	140 (38%)	-

2. MosquitoWise survey targeted at horse owners

2.1 Demographics

The horse owner-targeted survey was accessed 535 times during the sampling period. A total of 235 responses were excluded due to incompleteness (n=216), not owning or taking care of a horse (n=18) or living outside of the Netherlands (n=1). Full responses were obtained from 300 horse owners. Of the respondents, 279 (93%) were female and 14 (4.7%) were male.

Ages of respondents ranged from 18 to 86 years with a median age of 41, which is lower than the median age of the Dutch population (42.4) (144). Most respondents completed post-secondary education or higher (89%) and were employed when filling out the survey (79%). A large proportion (27%) of respondents were working as healthcare practitioners or technical and healthcare supporters at the time of the study, whereas the overall Dutch employment in healthcare is 16% (145). The majority of the survey's respondents lived in rural areas (68%), compared to only about 30% of the total Dutch population (146).

2.2 Knowledge and HBM scores

Knowledge scores ranged from 0 to 9, with a median of 6 and mean of 5.96. Twelve horse owners (4%) had a minimum knowledge level, 154 (51.33%) had an intermediate knowledge level and the remaining 134 (44.67%) had a high knowledge level. Scores for separate constructs and the total HBM score are shown in Table 3. The high 'cues to action' construct score indicates that cues have a positive effect on the horse owners' overall intent to use preventive measures. The perceived severity scores indicate that horse owners are aware that infections with MBVs can lead to (severe) disease in humans. The overall mean HBM score was 29.41. This suggests that horse owners have a moderate to moderately high level of intention to use prevention measures (to protect themselves) based on the HBM framework.

Table 3. Health belief model construct and total scores for horse owners. HBM: Health Belief Model. The construct score range is 1-7. The total HBM score range is 6-42.

Construct	Mean [25%-75%] Median score N = 300
Perceived susceptibility	4.34 [3.75-5.00] 4.25
Perceived severity	5.48 [4.67-6.00] 5.67
Perceived benefits	4.81 [4.33-5.33] 5.00
Perceived barriers	4.85 [4.00-6.00] 5.00
Cues to action	5.28 [4.67-6.00] 5.33
Self efficacy	4.65 [4.00-5.50] 4.75
HBM total score	29.41 [27.29-31.58] 29.46

2.3 Horse owner specific questions

Most horse owners were aware of the option to vaccinate their horse(s) against WNV (85%). However, just over half (50.67%) of the horse owners would consider to vaccinate or were already vaccinating their horse(s) yearly. Yearly vaccination was described as a double vaccination in the first year, followed by a yearly single vaccination of approximately €55, excluding call-out fees. Respondents were then asked whether they applied preventive measures against insect bites for their horse (not specifically against mosquitoes). For this question, the most frequently chosen multiple-choice options 'use of a fly mask' (72.33%), 'daily use of insect repellent spray' (54%) and 'use of a fly blanket' (57.33%). Additionally, 33 respondents used the 'other' textbox to describe their preventive measures. These responses included a wide range of answers, such as spraying insecticides in the stable, changing feed or supplements and encouraging natural enemies (e.g., *Hirundinidae* species). A few respondents gave answers unrelated to prevention of insect bites, such as vaccination and prednisone administration. Twenty-three horse owners (7.67%) did not take any preventive measures. Respondents were asked for which symptom(s) in their horse(s) they would contact their veterinarian. Answers to this question, shown in Figure 2, indicate that most horse owners would contact their veterinarian for symptoms such as fever and neurological symptoms. Only two respondents (0.7%) indicated they would not contact their veterinarian for any of the mentioned symptoms.

Social media (22.3%) and online equine fora (24.3%) (Bokt.nl in our case) were the most frequently mentioned sources of information for mosquitoes and MBVs. The (equine) veterinarian is another important information source (19.3%). Interestingly, 45.8% of horse owners did not recently read or receive any information about MBVs via any of the proposed information channels.

Figure 3 shows the 7-point Likert scale responses to the two remaining questions. A majority felt that their veterinarian was responsible for informing them about MBVs in horses. About half of the respondents removed breeding sites in the stabling area.

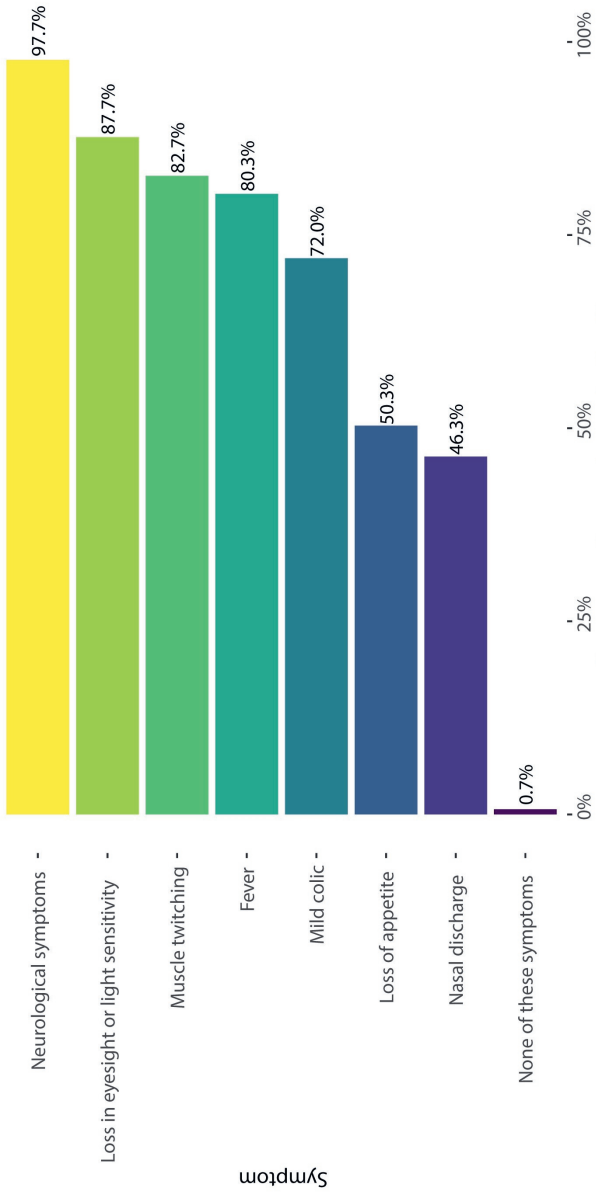


Figure 2. Bar plot of respondents' answers to the question "For which of these symptoms would you contact your veterinarian?". Neurological symptoms were described as weakness in hind limbs, paresis, paralysis and convulsions.

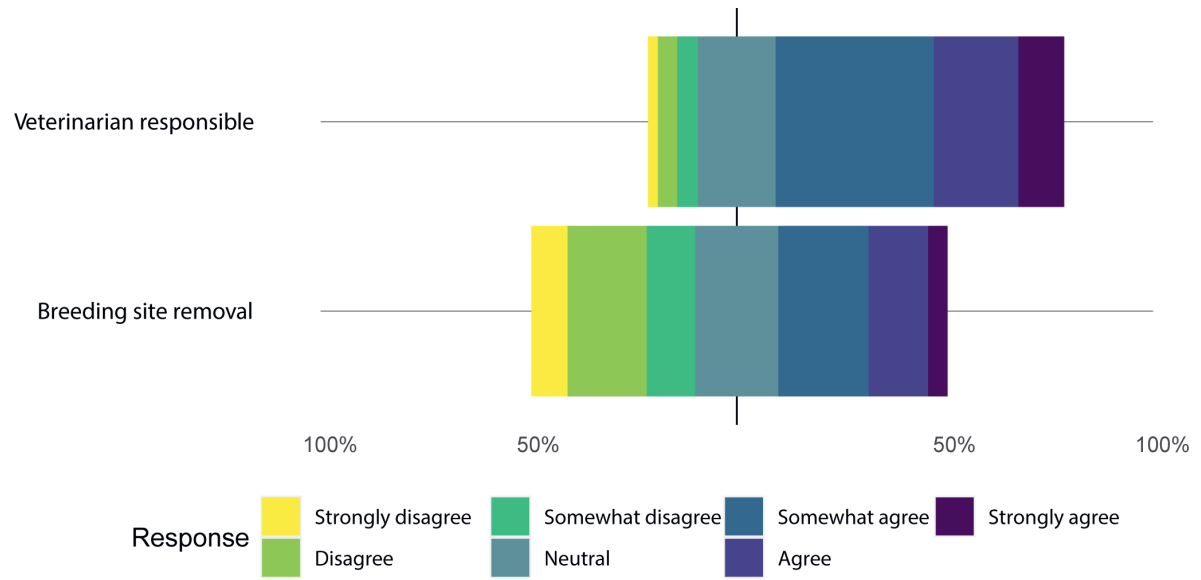


Figure 3. Likert scale results for horse owner questions: 'Veterinarian responsible' is short for "I think my veterinarian is responsible for informing me about mosquito-borne viruses in horses." And 'Breeding site removal' is short for "During mosquito season (March through September), I will ensure there are no mosquito breeding sites around my horse's stable".

Discussion

In this study, we investigated the seroprevalence of WNV and USUV in domestic horses and dogs and studied the MBV knowledge and perceptions of horse owners in the Netherlands. We found a low observed seroprevalence for USUV and WNV between May 2021 and May 2022. The MosquitoWise survey revealed a high knowledge level for horse owners and high awareness of WNV vaccination but a lower intention to vaccinate.

Serosurvey

The required sample size as calculated prior to the study was not met for both horses and dogs. Veterinarians received monthly reminders to submit samples, but many practices mentioned the effect of COVID-19 pandemic measures and personnel shortage on their ability to cooperate. Some equine veterinarians actively promoted WNV vaccination at the start of 2021, lowering the number of horses suitable for inclusion in the study.

This was the first study to investigate the seroprevalence of WNV and USUV in domestic animals in the Netherlands. In 2018, Zaaijer et al. (123) performed an USUV screening study in Dutch blood donors and found that 0.45% of the donors showed USUV IgG responses in September and a similar result was found in Dutch bird ringers in 2021 (147). Our findings are in line with both studies in humans. The low seroprevalence for USUV is interesting considering the circulation of the virus since 2016 and high impact on the bird community (55). Possible explanations for this finding are that antibody titres of our study animals had already waned or that animals were never infected, potentially due to housing conditions or specific host-preferences of vectors (53).

Horses are extensively used for WNV seroprevalence studies throughout Europe (148). In eastern Germany, Ganzenberg et al. (149) found a seroprevalence of 5.8% for WNV in horses in 2020. It is noteworthy that they also included animals with a foreign background. In eastern Austria, seroprevalence in horses was 15.5% for TBEV, 5.3% for WNV and 0% for USUV in 2017. Again, some horses had questionable countries of origin or travel history. Therefore, they also mention an 'autochthonous prevalence' of 1.2% for WNV (150). We also noted the exclusion of six horses as owners initially did not declare the right country of origin or vaccination status. This highlights the fact that our data, and also that from other studies, should be interpreted with caution. Equine WNV seroprevalence in Spain is much higher, namely 19.7% in western Spain (in 2018-2019) and even 25.0% (in 2020) in feral horses in the south-west (151,152). Multiple countries have used dogs to study WNV, TBEV and (to a lesser extent) USUV seroprevalence (132). In France, an USUV seroprevalence of 1.08% was found in dogs

between 2016 and 2020. This seroprevalence is slightly higher than our findings. In the same study, a higher seroprevalence was found for both USUV (3.83%) and WNV (13.19%) in horses (74). Differences in seroprevalence between countries and animal species can be explained by many factors, such as endemicity of the virus, sampling strategy, demographics and housing conditions of the animals. Furthermore, there are notable differences in types of diagnostic methods used, which hampers comparability of seroprevalence estimates between countries (132,148).

The majority of doubtful and positive ELISA sera turned out to be negative in all VNTs, while specificity is presumed to be high for horse sera (130). An explanation for this may be waned antibody titres below detection limits of the VNTs. Orthoflavivirus antibody persistence differs per virus and for each host species. Evidence suggests long term persistence of antibodies for WNV and TBEV in multiple species, including horses (153–155). No studies have been published on orthoflavivirus antibody waning in dogs. Furthermore, the higher starting dilution of TBEV compared to that of USUV and WNV might have resulted in false negative outcomes for sera in the TBEV VNT. Another explanation may be that these ELISA positives could be due to circulation of other viruses not tested for neutralizing antibodies in this study, such as BAGV or LIV. This was also suggested by a recent study performed by Laidoudi et al. (96) in France. However, published BAGV detections in Europe are limited to Spain and Portugal (156). Furthermore, there are no publications of BAGV infections in dogs and horses. LIV infections in horses have been described, but cross-reactions with WNV or USUV have not been investigated nor reported (157,158).

The owner questionnaire revealed interesting results on possible risk factors, even though a formal risk factor analyses could not be performed. The combination of a relatively high percentage (65%) of repellent use in dogs, combined with the limited amount of time they spend outside, may be the cause of the low seroprevalence observed in this study. Interestingly, dog owners reported more frequent tick removals (33%) than horse owners (9.6%). This observation may be explained by the fact that dog owners actively search for ticks, and dogs may spend more time in tick-infested vegetation compared to horses. Moreover, a smaller percentage of horse owners (35%) indicated that they used insect repellents compared to dog owners (65%). However, it should be noted that we did not ask owners what type of repellents were used. Repellency and efficacy differ per repellent type and, thus, may influence the results (159).

Other studies claimed the use of military or hunting animals as suitable species for arbovirus surveillance because of their frequent exposure to vectors (95–97,132). A significant difference between seroprevalence in pet dogs and hunting dogs was found

by García-Bocanegra et al. (132) in Spain. Future studies to investigate the seroprevalence of orthoflaviviruses in the Netherlands could focus on animals that spend more time outdoors or are used for hunting or military work. Another option would be to investigate the potential of other animal species, such as (captive) birds, e.g. chickens, goat, cattle or wildlife.

MosquitoWise

The MosquitoWise survey in horse owners revealed a higher mean knowledge score compared to the Dutch general public. Abourashed et al. (140) found a mean knowledge score of 4.34, while the horse owner mean score was 5.96 on a scale from 0 to 9. Additionally, all mean and median scores for separate constructs were higher, except for the perceived benefits of prevention measures. The higher perceived reversed barriers construct score implies horse owners experience lower barriers to use prevention measures, compared to the Dutch panel. The total mean HBM score in horse owners is 1.3 points higher than in the Dutch panel, which indicates that horse owners have more health belief and more intent to show preventive behaviour (140). Scores may be influenced by the fact that horse owners are a specific subpopulation of the Dutch general public. Of our respondents, 93% were female, and the median age of 41 was lower compared to the Dutch median of 49. The majority live in rural areas (68%) and a substantial proportion works in healthcare related professions. The already mentioned bias in gender, age and profession is also found in other studies from the UK (116,160).

There are few published studies on knowledge or attitudes towards MBVs in horse owners in Europe. Chapman et al. found that 65.4% of horse owners correctly identified mosquitoes from an image (116). The majority (83.1%) of their respondents were aware that WNV can affect horses, which is similar to the percentage of respondents in our study (85%) when asking about awareness of the possibility of WNV vaccination. Horse owner intent to vaccinate against WNV was much higher (80.1%) compared to our results (50.67%), but it should be noted that we did not ask specifically for vaccination intent 'in the case of a WNV outbreak event' in contrast to their study. Factors limiting willingness to vaccinate can be related to concerns about side effects and efficacy, lack of risk of disease and associated cost, among others (116,161).

Owners feel their veterinarian is responsible for providing them with information about MBVs, which also aligns with earlier research (116). Most horse owners will contact their veterinarian if their horse shows symptoms associated with WNV infection, such as fever, neurological symptoms and muscle twitching. This contact between owner

and veterinarian may aid in early detection of WNV cases. However, this also requires awareness of, and rapid notification of, suspected cases by veterinarians.

Conclusions

The low seroprevalences of WNV and USUV indicate many dogs and horses remain susceptible, offering opportunities for trend analysis and surveillance. However, despite multiple recent detections of WNV, USUV, and TBEV in humans, the role of dogs and horses in early detection of human cases is debatable. High awareness among horse owners and the absence of detected equine WNV cases highlight this uncertainty. Continued surveillance is crucial for detecting increased virus circulation and protecting both animal and human health. Additionally, veterinarians' awareness and intentions to notify suspected cases need further investigation.

Acknowledgements

The authors acknowledge Jenny Hesson, Åke Lundkvist and Reina Sikkema for critically reviewing the manuscript. Additionally, we thank Kees van Maanen (Royal GD) for providing us with WNV reference sera and the Virology department of Wageningen University & Research for the WNV strain used in the virus neutralization test. The authors thank all participating veterinarians and horse and dog owners for participating in this study.

Supplementary material

Supplemental material A. Serosurvey data – Information and test results obtained in the serosurvey of horses and dogs

Available through:

Supplemental material B. Translated submission form serosurvey participants

Information sampled animal:

Housing conditions (during mosquito season April-November)*

Practice name*				
Postal code veterinary practice*				
Animal information				
Name of the animal				
Postal code place of residence animal*				
Municipality place of residence animal*				
Birth date* (minimum 1 year old)				
Sex*				
Breed (in case known)				
Sampling date*				
What is the area of residence of the animal?	Urban area	Agricultural area	Nature area	
How many hours does the animal spend outside per day?	0-6 hours	6-12 hours	12-18 hours	More than 18 hours
Do you use an insect repellent (like a spray, collar or pipette)?	Yes		No	
Have you removed a tick from your animal during the past 12 months?	Yes		No	
Please only respond to the questions below when the submitted sample is from a horse				
How does the horse get turned out during this period?	Pasture	Paddock	Both	
Do you use a blanket for the horse during this period?	Yes		No	
What is the number of horses housed at the premise where the horse is stabled?	1-2	3-10	10-20	>20

*mandatory field

Supplemental material C. Translated MosquitoWise questionnaire addition specifically directed to horse owners and caretakers

Section J: part 10. Horses and mosquitoes
In this part of the survey we will ask you some questions about mosquito-borne viruses in horses.

J1. Have you ever heard about the possibility to vaccinate your horse(s) against West Nile Virus?

- ☐ Yes
- ☐ No

J2. I would consider yearly vaccinating my horse(s) against West Nile Virus (or have already done so)
*Vaccination of your horse costs approximately 55 euros per year (excluding call-out fees)

- ☐ Yes
- ☐ No

J3. Which preventive measures do you take to protect your horse(s) against insect bites* during the mosquito season (March through September)
*This question is about all preventive measures against insects (including mosquitoes)

- ☐ Use of insect repellent spray (daily)
- ☐ Use of a fly blanket
- ☐ Use of a fly mask
- ☐ Use of a ventilator inside the barn/stable
- ☐ Removal of possible breeding sites in and around the stable/premise
- ☐ Stabling my horse(s) during sunrise and sunset
- ☐ I do not take any preventive measures
- ☐ Other: …

J4. During mosquito season (March through September), I will ensure there are no mosquito breeding sites around my horse's stable

- ☐ Strongly agree
- ☐ Agree
- ☐ Somewhat agree
- ☐ Neutral
- ☐ Somewhat disagree
- ☐ Disagree
- ☐ Strongly disagree

J5. For which of the following symptoms in your horse(s) would you contact a veterinarian?

- ☐ Fever

- Neurological symptoms (weakness in hindlimbs, paresis, ataxia paralyses, convulsions)
- Loss of appetite
- Muscle twitching
- Loss in eyesight or light hypersensitivity
- Nasal discharge
- Mild colic
- None of these symptoms

J6. I think my veterinarian is responsible for informing me about mosquito-borne viruses in horses.

- Strongly agree
- Agree
- Somewhat agree
- Neutral
- Somewhat disagree
- Disagree
- Strongly disagree

J7. Have you recently read or heard information about mosquitoes or mosquito-borne viruses in horses from one of these sources?

- (equine) veterinarian
- Horse magazine (Bit, Hippische Ondernemer etcetera)
- Horse- or laboratory organisation (KWPN, KNHS, GD Deventer)
- Online horse forum (bokt.nl)
- Government website
- Social media (e.g. Instagram, Twitter, Facebook, YouTube, online news articles)
- Family and friends
- Educational organisations (e.g. school or university)
- Institutional websites (e.g. WHO, ECDC, WOAH)
- Television and news channels
- Printed newspaper
- Radio
- I have not seen or heard information from one of these channels
- Other: ....

Thank you for filling out this survey, your input is much appreciated. Even though mosquito-borne viruses are not very prevalent in Europe, we have asked you many questions about (biting)mosquitoes and mosquito-borne viruses. If you want to know more about this topic or if you do have any questions after filling out this survey, please visit our information page: LINK

If you want to know more about mosquito-borne viruses in horses, please have a look at this website: LINK

Supplemental material D. Sample sizes and excluded animals for each step of the serosurvey

Table SD1. *Sample size, tested and excluded animals per species. Number of excluded animals are underlined*. NA*: not applicable.*

Species	**Animals sampled**	**Competition ELISA** (positive/tested)	**IgM WNV ELISA** (positive/tested)	**Animals excluded**	**Animals included** (see manuscript Table 2)	**Insufficient serum to perform VNT**	**VNT** (positive/tested, see manuscript Table 1)	**Cytotoxic in VNT(s)**	**Positives** (VNT positive/total)
Horse	370	18/370	0/18 (competition ELISA +) *0/166 (competition ELISA -)*	6/18*	364	0/12	1/12	1/12	WNV: 1/363 USUV: 0/363
Dog	258	7/258	NA	0/258	258	1/7	2/6	0/6	USUV: 1/257 WNV: 0/257 Undetermined**: 1/257

* Four of the (18) ELISA positive horses appeared to have been vaccinated even though owners initially declared they had not been vaccinated as a prerequisite to be eligible to participate in the study. One horse was accidentally vaccinated once 8/9 years ago, the owner was unsure with which vaccine. One horse was vaccinated in 2012. One horse was vaccinated against WNV one year ago. The fourth horse had been vaccinated with an unknown vaccination date.
Furthermore, two horses appeared to have been abroad, even though owners initially declared they had not been. One of these horses originated from Ireland and had been to Belgium, while the other originated from Austria.

**Similar neutralizing titres for WNV and USUV

Chapter 3.

Sindbis virus (SINV) in the Netherlands: Evidence for local circulation in wild birds and horses

Kiki Streng[1]*, Cora M. Holicki[2]*, Jenny C. Hesson[3,4], Heather Graham[5], Felicity Chandler[2], Louie Krol[6], Rody Blom[7], Emmanuelle Münger[2], Anne van der Linden[2], Constantianus J.M. Koenraadt[7], Maarten Schrama[6], Chiara de Bellegarde de Saint Lary[8,9], Leo G. Visser[8], Bas Oude Munnink[2], Åke Lundkvist[3], Marion P.G. Koopmans[2], Henk P. van der Jeugd[10], Wim H.M. van der Poel[1,4]*, Reina S. Sikkema[2]*

* Authors contributed equally

Submitted

Affiliations
[1] Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands
[2] Viroscience, Erasmus Medical Center, Rotterdam, the Netherlands
[3] Department of Medical Biochemistry and Microbiology, Zoonosis Science Center, Uppsala University, Sweden
[4] Biologisk Myggkontroll, Nedre Dalälvens Utvecklings AB, Gysinge, Sweden
[5] Wageningen Bioveterinary Research, Lelystad, the Netherlands
[6] Institute of Environmental Sciences, Leiden University, Leiden, the Netherlands
[7] Laboratory of Entomology, Plant Sciences Group, Wageningen University, Wageningen, the Netherlands
[8] Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
[9] Julius Centre for Health Sciences and Primary Care, Department of Epidemiology, University Medical Center Utrecht, Utrecht, the Netherlands
[10] Dutch Centre for Avian Migration and Demography, NIOO – KNAW, Wageningen, the Netherlands

Abstract

We report first evidence for Sindbis virus (SINV) circulation in the Netherlands. Serological evidence of SINV infections was found in twelve wild birds and three horses (January 2021 - December 2022). SINV was also molecularly detected, and a partial genome was obtained from a European robin sampled in October 2022.

Introduction

Sindbis virus (SINV; *Togaviridae*; *Alphavirus*) is maintained in an enzootic transmission cycle between birds (e.g., passerines and grouse) and mosquito vectors (mainly *Culex,* but also *Aedes* and *Culiseta* spp.) (162). SINV has been detected throughout Eurasia, Africa, and Australia. Horses and humans are considered dead-end hosts. Clinical cases in humans are commonly reported in Northern Europe (Finland and Sweden) and South Africa (163,164). The majority of human SINV infections are considered to be asymptomatic or mild, associated with skin rash, muscle pain, and/or fever, but some patients develop arthralgia and (severe) polyarthritis (162). Infections in non-symptomatic horses have been described in Sweden through the detection of neutralizing antibodies (106), but SINV infections can also result in neurological signs in horses as shown in South Africa (165). Evidence of exposure in wildlife has been reported in many European countries, including Germany and the United Kingdom, but not the Netherlands (163). Therefore, the presence of SINV was investigated in the Netherlands in mosquitoes, birds, horses, and humans.

The study

Throughout the Netherlands, mosquitoes, wild birds, horses, and humans were screened for SINV RNA and/or neutralizing antibodies (Figure 1).

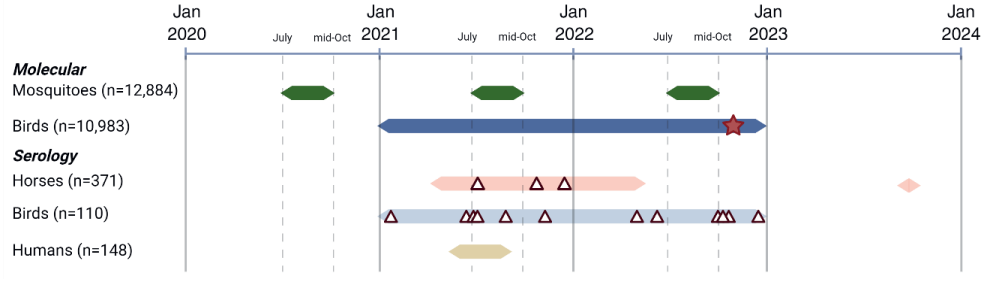


Figure 1. Timeline of sampling and Sindbis virus (SINV) findings per animal group. Red star (SINV-RNA) and triangles (SINV-antibodies) indicate positive samples. Created with BioRender.

Mosquitoes (n = 12,884) were sampled throughout the Netherlands from July to mid-October in 2020, 2021, and 2022 as previously described (35). Live wild birds (n = 10,983) were sampled (throat and cloaca swabs, serum, and feathers) in 2021 and 2022 as part of ongoing research into arbovirus dynamics in birds (58). Sera of horses (n = 368) were collected between May 2021 and May 2022 (166), with additional sera (n = 3) collected in October 2023. Sera from bird ringers (n = 148) were collected from June to September 2021 as previously described (147).

Molecular analyses

All mosquitoes and birds were tested for SINV RNA by real-time reverse transcription (RT) PCR (detailed method in supplementary material). All mosquito pools tested negative for SINV RNA (Figure 2A; Figure S2). Of the live bird samples screened (9,599 birds caught/tested once and 593 birds caught/tested repeatedly), SINV RNA (pooled swab samples and feather) could be verified in one adult European robin (*Erithacus rubecula*) caught in Naarden, Noord-Holland (23.10.2022) (Figure 2B; Figure S2).
A partial SINV sequence was generated from the isolate by Sanger (172 bp fragment) and amplicon-based nanopore sequencing (245 bp fragment) from the open reading frame 1, NSP3 segment (GenBank accession no. requested); Figure 3; detailed method in supplementary material). The partial sequence from the NSP3 region was used for the phylogeny and shown to cluster with a SINV genotype I sublineage with sequences from Germany, Nordic countries (Finland, Sweden, and Norway), and Russia (99% identity). Virus cultivation of the isolate on mammalian (Vero ATCC, Vero E6) and mosquito (C6/36; *Aedes albopictus*) cells was unsuccessful. The robin had already been caught one year prior (31.10.2021) at the same site yet had not been sampled.

Serological analyses

A total of 368 horses were initially tested for SINV neutralizing antibodies by a Plaque Reduction Neutralization Test (PRNT) (supplementary material figure S1; (167)). Positive samples in the pre-screening were end-titrated. Three mares (0.82%, 3/368; 95% CI 0.28%–2.37%) sampled between July and December 2021 were seropositive, with titres ranging from 20 to 80 (Figure 2D and Table 1). Horse A and C did not have a history of clinical signs. Horse B, with the highest $PRNT_{80}$ titre (80), started to show neurological signs in September 2021 and was sampled in October 2021. Clinical signs consisted of posterior paresis, reduced speed and instability at replacement in foot-placing tests as well as falling backwards and into seated position. Due to the prolonged duration of disease, the horse was euthanized. Three additional horses from the same premise as horse B were sampled in October 2023 to investigate additional infections at this location, but all were PRNT negative. None of the horses travelled outside of the Netherlands.

Wild bird sera within a 16 km radius to the three seropositive horses and the RT-PCR-positive bird were examined by PRNT (2021-2022; n = 110). 10.91% (12/110; 95% CI 6.35%–18.10%) of these bird sera were seropositive (Figure 2D and Table 1). Of the 110 bird samples tested four samples were derived from two birds (Table 1). Neutralizing antibodies were found in common blackbirds (*Turdus merula*; n = 10) and song thrushes (*Turdus philomelos*; n = 2). The titres varied between 20 and 320 $PRNT_{80}$. All the 148 human bird ringer sera tested were SINV seronegative (Figure 2C).

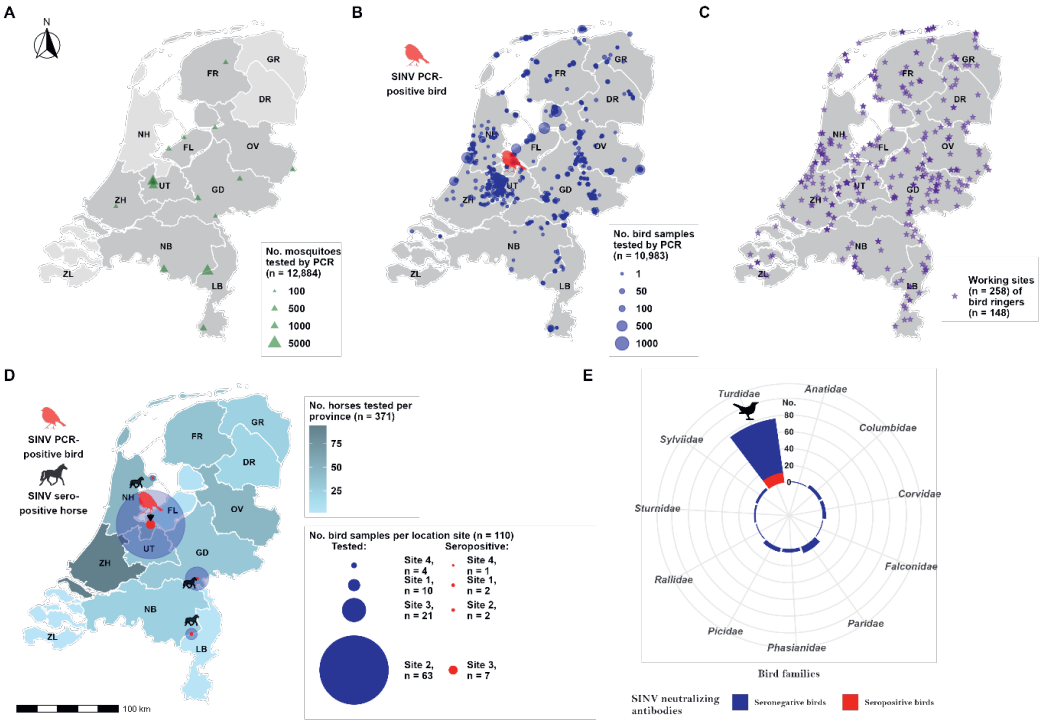


Figure 2. Maps showing the capture sites and the number of mosquitoes (A), and bird samples (B) screened for Sindbis virus (SINV) by RT-PCR throughout the Netherlands. (C) Bird ringing sites (n = 258) of the bird ringers (n = 148) screened for SINV neutralizing antibodies throughout the Netherlands. (D) Overview of the serological screening of horses throughout the Netherlands and birds in a 16 km radius to SINV-findings (bird icon: RT-PCR-positive bird; horse icons: seropositive horses). (E) Circular bar chart showing family distribution of the bird samples tested and positive for SINV neutralizing antibodies (n = 110). Province abbreviations: Drenthe (DR), Flevoland (FL), Friesland (FR), Gelderland (GD), Groningen (GR), Limburg (LB), Noord-Brabant (NB), Noord-Holland (NH), Overijssel (OV), Utrecht (UT), Zeeland (ZL), and Zuid-Holland (ZH).

Figure 3. Phylogenetic tree based on the obtained partial SINV sequence highlighted in yellow (245 bp; region: open reading frame 1, non-structural protein 3 (nsP3)) from the European robin from the Netherlands. Tip colours correspond to location of sequence and node shapes are coloured according to bootstrap values. Horizontal branch lengths are drawn to scale (the scale bar represents 0.1 nucleotide substitutions per site); vertical separation is for clarity only.

Table 1. Summarizing the metrics of the seropositive horses and birds and their neutralizing antibody titres as determined by $PRNT_{80}$. F: female, M: male, NA: not applicable.

Site (Figure 2D)	Animal ID	Sampling date	Species; Breed	Age (years)	Sex	$PRNT_{80}$ titres	Clinical signs
1	Horse A	31.07.2021	Horse A (*Equus caballus);* Dutch Warmblood	10	F	20	No
3	Horse B	15.10.2021	Horse (*Equus caballus);* Friesian	1	F	80	Yes, neurological
4	Horse C	09.12.2021	Horse (*Equus caballus);* New Forest pony	30	F	20	No
4	L582243	14.11.2021	Common blackbird (*Turdus merula*)	>1	F	320	NA
3	L571081	28.09.2021	Common blackbird (*Turdus merula*)	1	M	320	NA
3	H363127	22.06.2022	Song trush (*Turdus philomelos*)	>2	M	320	NA
3	L521438	21.06.2021 23.10.2021	Common blackbird (*Turdus merula*)	<1	M	40 (21.06.2021) <20 (23.10.2021)	NA
2	L586249	01.05.2022	Song trush (*Turdus philomelos*)	>1	M	80	NA
3	L521227	29.06.2021	Common blackbird (*Turdus merula*)	4	F	20	NA
3	L571047	30.06.2021	Common blackbird (*Turdus merula*)	>2	M	20	NA
3	L521403	06.01.2021	Common blackbird (*Turdus merula*)	2	M	40	NA
3	L521265	16.10.2022	Common blackbird (*Turdus merula*)	1	M	160	NA
2	L586400	23.10.2022	Common blackbird (*Turdus merula*)	2	M	40	NA
1	L606047	27.12.2022	Common blackbird (*Turdus merula*)	>1	M	20	NA
1	L590812	19.10.2022	Common blackbird (*Turdus merula*)	>1	F	40	NA

Discussion

This study describes for the first time the circulation of SINV in the Netherlands, as verified by the detection of RNA in a European robin as well as seropositive common blackbirds, song thrushes, and horses in distinct geographical regions. Seropositive birds caught in May and June (two song thrushes and three blackbirds) as well as one caught multiple times within one season (one blackbird) are considered Dutch breeding birds and likely acquired an infection with SINV locally. However importation cannot be fully ruled out as the seropositive song thrushes may have overwintered in Southern Europe. The other six seropositive blackbirds and the PCR positive European robin were caught during autumn and can be migrants from Northern Europe and hence, for them an exact location of infection cannot be determined (168). The generated partial sequence suggests that SINV has been introduced into the Netherlands from neighbouring countries in Europe either from Germany or the North (e.g., bird migration from Nordic countries). However, more (whole-genome) sequences from the Netherlands and other countries would be required to make conclusions over the exact route of SINV introduction.

It is important to consider SINV in the differential diagnosis of neurological signs in horses, as was done in South Africa where multiple infections were linked to SINV (165). In this study, prolonged neurological signs were observed in one horse even though it was not possible to confirm that the clinical picture resulted from a SINV infection. In Sweden seropositivity was only noted in horses with an unknown clinical history (106).

In line with previous studies in the Netherlands, SINV was not detected in mosquitoes (169,170). Most of the mosquitoes screened belonged to the species *Culex pipiens* or *Culex torrentium*, which are known to be competent vectors for SINV (162). SINV has been isolated from 11 mosquito taxa in neighbouring Germany in the past (171,172). However, high infection rates are not expected in the Netherlands, similarly to Germany, where only 21 of 22,528 mosquitoes (2019-2021) were positive for SINV (172). It must be noted that mosquito trapping was not focused on the sites where SINV antigen or antibodies had been detected in the birds or horses.

This study did not detect SINV seropositivity in humans and neither have human SINV infections been reported so far in the Netherlands. This may either be due to limited awareness and therefore no testing, or absence of spillovers into the human population, and more detailed studies are needed to assess possible human impact. Unlike in Finland, SINV infections are not notifiable in the Netherlands. In Finland, surveillance studies over the past decades suggest a rise in the number of human infections (162).

In conclusion, the findings described in this paper highlight the importance of surveillance for SINV as well as the need for increased awareness among Dutch veterinarians and health practitioners.

Acknowledgements

We are grateful to Irina V. Chestakova, Marjan Boter, Isa Meeuwissen, Babette Weller, and Merve Tok for their contribution to sample processing and testing. We also want to thank Tijs van den Berg, Natasja van Nijen, and Jurrian van Irsel for their assistance in sample and metadata collection for the birds. Furthermore, we would like to thank all horse owners and veterinarians for their cooperation in this study. This work was supported by the Dutch Research Council (project 'Preparing for vector-borne virus outbreaks in a changing world: a One Health Approach'; NWA.1160.1S.210) and the European Union's Horizon 2020 research and innovation programme under Grant No. 874735 (VEO).

Ethics approval statement

Horses in this study were sampled under legislation (license no. AVD40100202114384) of the Dutch Central Authority for Scientific procedures on Animals (CCD). Sampled birds were ringed and sampling was performed under ethical permit AVD801002015342 and AVD80100202114410 issued to NIOO-KNAW. The sampling of the bird ringers was approved by the Medical Research Ethics Committee of Leiden, The Hague, Delft in the Netherlands (number P20.112).

Supplementary material

Serological analysis

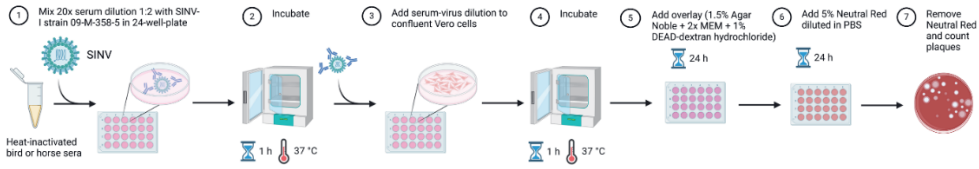


Figure S1. Workflow for testing horse and bird sera by means of a Plaque Reduction Neutralisation Test (PRNT) for Sindbis virus (SINV). MEM: Minimum Essential Medium, DEAD: Diethylaminoethyl, PBS: Phosphate-Buffered Saline. Created with BioRender.

Molecular analysis

For molecular analyses mosquito samples were species determined and pooled either at Leiden University (2020) or at Centrum Monitoring Vectoren (CMV), Netherlands Food and Consumer Product Safety Authority (NVWA) (2021 and 2022) as described earlier (169). Subsequently, the mosquito pools were transported to Erasmus MC for molecular analyses. Morphological identification of mosquitoes was performed as described by Becker et al. (173). The mosquitoes were pooled (maximum pool size was 10 individuals) according to species level, sampling location, and time point (monospecific species pools belonging to the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex,* and *Culiseta* and mixed pools with *Cx. pipiens/torrentium*). The pools were homogenised in 1 ml medium (DMEM, NaHCO3, Hepes-buffered saline, penicillin/streptomycin, amphotericin). RNA was extracted from the homogenised mosquito pools by manual extraction with AMPure XP Beads (Beckman Coulter, Brea, CA, United States of America). Wild bird screening for SINV RNA was performed on swabs (throat or cloacal) collected during the capturing and ringing of the birds. RNA was extracted from the bird swabs and feathers using the Viral NA Large Volume Kit and MagNA Pure 96 System (Roche Holding, Basel, Switzerland). The mosquito/wild bird eluate was tested by means of a SINV-specific real-time RT-PCR described by Sane et al. (174) (NS1: forward primer: GGTTCCTACCACAGCGACGAT, reverse primer: ATACTGGTGCTCGGAAAACATTCT, and probe: TTGGACATAGGCAGCGCACCGG) using the LightCyler 480 (Roche LifeScience, Basel, Switzerland). Samples that were tested positive were confirmed in a second PCR targeting a different region of the genome (NS2: forward primer: CGTCGAAGACAGTAGATTCGGTTA, reverse primer: CGCGAACGCTTCGTCAA, and probe: TCAACGGATGCCACAAAGCCGTAGAA) as well as being subjected to sequencing.

Partial sanger sequencing of SINV PCR products (forward primer: CATAACCCGTCGTCTAGC and reverse primer: TAGGCTGTTCTGGCACTT) was

performed in addition to whole genome sequencing using an amplicon-based Oxford Nanopore MinION (Oxford Nanopore technologies, https://nanoporetech.com) approach using modified primers from (59). An alignment was generated using AliView (175) and 73 sequences downloaded from BV-BRC (https://www.bv-brc.org) or the GenBank data base (https://www.ncbi.nlm.nih.gov/genbank). From the alignment a maximum-likelihood phylogenetic analysis was conducted using the best-fitted nucleotide substitution model (GTR+F+I+G4) in the IQ-TREE web server (176). The resulting maximum-likelihood phylogenetic tree was visualized and edited with R 4.3.2. (177) using the packages ggplot2 (v 3.5.1, (178)) and ggtree (v 3.10.1, (179)) and revised using Inkscape 1.3.2.

Virus cultivation of the SINV-positive isolate on mammalian (Vero ATCC, Vero E6) and mosquito (C6/36; *Aedes albopictus*) cells did not result in a CPE and the supernatant was PCR-negative 1-, 2-, 3-, and 8-days post infection.

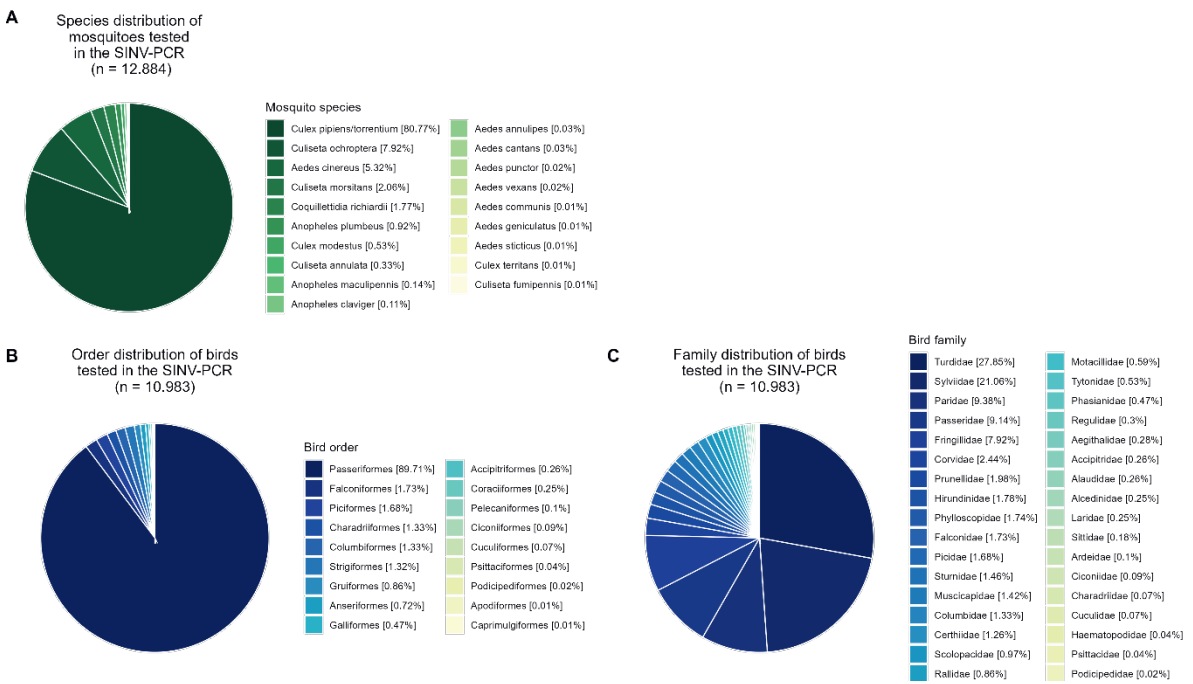


Figure S2. Pie charts portraying mosquito species distribution (A) and bird order (B) and family (C) distribution of samples screened for Sindbis virus by PCR.

Chapter 4.

Evidence of West Nile virus (WNV) infections in wild boars (*Sus scrofa*) in the Netherlands, 2018-2021

Kiki Streng[1], Eduardo de Freitas Costa[2], Sophie van Oort[2], Renate Hakze-van der Honing[2], Gebbiena Bron[3], Carl Andreas Grøntvedt[3], Wim H.M. van der Poel[1,2], Nathanaël Hozé[4,5,6], Michel Counotte[2]

In preparation

Affiliations
[1] Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands
[2] Wageningen Bioveterinary Research, Lelystad, the Netherlands
[3] Norwegian Veterinary Institute, Norway
[4] Université Paris Cité, INSERM, IAME, F-75018, Paris, France
[5] Institut Pasteur, Epidemiology and Modelling of Antimicrobials Evasion research unit, Paris, France
[6] Université Paris-Saclay, UVSQ, Inserm, CESP, Anti-infective evasion and pharmacoepidemiology research team, Montigny-Le-Bretonneux, France

Abstract

Orthoflaviviruses are emerging in Europe and have potential significant impact on animal and public health. Usutu virus (USUV) and West Nile virus (WNV) have both been detected in the Netherlands since 2016 and 2020, respectively. Interpretation of orthoflavivirus serology is complicated by cross-reactivity, among other factors. This study aims to provide epidemiological insights of USUV and WNV infections in wild boars in the Netherlands over the years 2018-2021. We further explore the interpretation of serological results, taking into account cross-reactivity patterns.

A total of 1,504 wild boars were tested using a screening competition ELISA, after which positive samples were confirmed using Virus Neutralization Tests for WNV and USUV. We explain the observed serology of the individuals with a 3-level Bayesian hierarchical model that jointly estimates (1) the probability of infection by WNV and USUV, (2) the antibody response upon infection, the impact of cross-reactivity on the antibody titers, and (3) the observation errors.

In total, 30.65% of sera had a doubtful or positive ELISA result. A declining trend in ELISA positives was observed over time. Based on the model outcomes, the probability of infection prior to sampling of WNV was highest in 2019 (13%) and lowest in 2020 (7.8%). Both the classical interpretation (four-fold titer difference as confirmation of infection) as well as the model results indicate a probability of infection of WNV in or before 2018 of at least 7%, thus providing robust evidence of WNV circulation in or before 2018. For USUV, model results show a much higher probability of infection (24.0%) in 2018 compared to the classical interpretation (7.8%). The estimated probabilities follow similar trends but are higher for both viruses and all years in the model results compared to the classical interpretation, especially for USUV in 2018. The probabilities of observing false positives and negatives were higher for WNV (9.1% and 10.4%) compared to USUV (2.0% and 3.0%). The sensitivity analysis highlights the need to account for cross-reactions. It confirms that the model deepens the understanding of the different processes that lead to the observations in the population.

1. Introduction

Orthoflaviviruses are emerging in Europe and have potential significant impact on animal and public health. West Nile virus (WNV) and Usutu virus (USUV) are orthoflaviviruses from the genus *Orthoflavivirus* in the family *Flaviviridae* (12). Both are transmitted by mainly *Culex* spp. mosquitoes and maintained in an enzootic cycle between (wild) birds and mosquitoes (180). Infections of these orthoflaviviruses in humans often result in asymptomatic or mild disease. However, WNV infections may cause significant disease and even mortality in a small proportion of infected individuals (62). In other mammals, infections often go unnoticed except for WNV infections in horses (30). Both viruses are currently co-circulating in Europe (117). In 2016, USUV was detected in the Netherlands and has been circulating since (55,56). WNV was first detected in 2020 in birds and mosquitoes (58). That year, eight autochthonous human cases were identified (57). The virus was detected again in a single bird in 2022 (126).

As viremia is cleared rapidly, virus detection in dead-end hosts is often unsuccessful, and thus serological methods must be used. However, co-circulation of orthoflaviviruses hampers diagnosing infections. Antigenic cross-reactivity complicates the interpretation of serological studies as viruses within the same serocomplex (117). WNV and USUV are both part of the Japanese Encephalitis serocomplex. Tick born encephalitis virus (TBEV), another orthoflavivirus that is also present in Europe, is part of a different serocomplex and antibodies are thus not expected to cross-react in VNTs. Generally, sera are first screened using an antibody binding approach such as an ELISA or Protein Microarray. Neutralization assays, such as a Virus Neutralization test (VNT) or Plaque Reduction Neutralization test (PRNT) are regarded as the gold standard to confirm infection (30). A four-fold VNT titer difference between different orthoflaviviruses is usually regarded as a determining factor for confirmation of past infection (30).

Interpretation of neutralizing titers is further complicated by factors such as individual variation in antibody development, waning of antibodies over time, coinfections, reinfections and others (68,117). This may lead to incorrect estimations and the inability to distinguish infections in individuals and thereby incorrect prevalence estimations. Diagnostic algorithms may guide interpretation of currently available serological tests (128,181). Development of more specific diagnostic tests is another, challenging, area of focus for research. Until these tests exist, other approaches must be explored to deal with serological interpretation of orthoflavivirus infections. One approach is statistical modelling of serological results in populations which explores circulation of multiple pathogens in the same population, considering co-circulation

and cross-reactivity. Studies using this approach have been published for pathogens like influenza A virus, chikungunya and o'nyong-nyong virus, and SARS-CoV-2 (182–184).

Wild boars (*Sus scrofa*) are suggested to be good species for orthoflavivirus surveillance, as they experience high exposure to mosquito bites, live in nature and forest rich areas (131,185,186). The Netherlands has an ongoing surveillance program set up in wild boars, to remain a free status for Classical and African Swine Fever, as well as other diseases (187). The study presented here aims to provide epidemiological insights of USUV and WNV infections in wild boars in the Netherlands over the years 2018-2021. We further explore the interpretation of serological results, taking into account cross-reactivity patterns, of these two viruses in wild boars and provide suggestions on how our findings may be incorporated in current and future surveillance programs.

2. Materials and methods

2.1. Study area and sampling

Wild boar (*S. scrofa*) samples used in this study were collected as part of an existing national monitoring program for swine diseases. Wild boar habitats are widely present throughout mainly the South-East of the Netherlands and the number of boars has been rising over the past decade. In a few areas wild boars can roam freely, such as 'De Hoge Veluwe' National Park and two nature areas in the province of Limburg. In all other areas, the Netherlands has implemented an active reduction policy, executed by hunters. Serum was taken by hunters after shooting (cardiac puncture) and was sent to the laboratory of Wageningen Bioveterinary Research in Lelystad. Demographic data were provided by the hunters via a submission form in most cases. Age in months was estimated by assessment of the teeth. The required sample sizes were calculated to detect a seroprevalence of 1% with a confidence level of 95% and a precision of 99% (81,141). A random selection of about 380 sera was included for each year.

2.2. Serology

All samples were first screened for orthoflavivirus antibodies by a commercial species-independent ELISA according to the manufacturer's protocol (ID Screen®, West Nile Competition Multi-species, IDvet Innovative Diagnostics, France). This ELISA is known to show cross-reactive results for WNV, USUV as well as TBEV in animals (130). Doubtful and positive sera were subsequently tested in duplicate in Virus Neutralization Tests (VNTs) for WNV and USUV according to a previously described method (166). Sera from an experimentally WNV infected horse and a field-infected USUV owl were used as positive controls. Forty-one sera with negative ELISA results

were randomly selected to be tested in VNTs, in order to assess the possibility of false positives in VNT, assuming a perfect sensitivity of the ELISA. Two additional sets of negative control populations were used to estimate the VNT cut-offs. Firstly, slaughterhouse samples of domestic Dutch pigs (n = 14) were used. Secondly, a set of sera from Norwegian wild boars (n = 50) hunted in 2018 and 2019 were used as negative control population as both USUV and WNV have not been detected in Norway (188). ELISA and VNT results of the Norwegian boars and domestic Dutch pigs are shown in supplementary material 1, Figure S1 and S2. We assumed no cross-reactivity to TBEV in VNTs, and therefore only WNV and USUV titers were taken to the model. Titers were read as the reciprocal of the highest dilution for which at least 50% cytopathic effect (CPE) was observed. All sera were tested in duplicate, and outcomes of both replicates were registered individually. A proportion of sera were of very bad quality and could either not be tested or CPE could not be interpreted in one or more VNTs. Therefore, these sera were removed from further analyses and not included in the statistical model. Neutralizing titers ≥10 were regarded positive for USUV, for WNV a neutralizing titer of ≥30 was regarded positive.

2.3. Statistical model of antibody response, viral circulation and measurement

We explain the observed serology of the individuals in the survey with a model that jointly estimates the probability of infection by WNV and USUV, the antibody response upon infection, the impact of cross-reactivity on the antibody titers and the observation errors.

Notations

All animals were individually identified by a unique number. All animals are assumed to be independent of each other. Demographic information and test results were stored in Excel (version 2308). VNT results can take the values (10, 30, 90 or ≥270) which we write with an exponent form $10*3^{o-1}$ for USUV and $10*3^{o}$ for WNV. All titers are thus given a label which can be: o = 1, 2, 3, 4 for USUV and o = 1, 2, 3 for WNV. VNT titers of 10 for WNV and of <10 and <30 (where the 1:10 dilution was not interpretable) for both USUV and WNV are given label 0 (negative). As all individuals were tested in duplicate in both VNTs, we write $Obs^W = (o_1^W, o_2^W)$ and $Obs^U = (o_1^U, o_2^U)$ for the set of observed WNV and USUV titers, respectively.

Hierarchical modeling

We built a 3-level Bayesian hierarchical model based on Hozé et al. (183), including the (1) infection and (2) antibody response models and extending the model with another level called (3) the observation model. The observation model takes into account the observational error due to an imperfect diagnostic method. Assuming the infection history (I_j) and the real titers (r_j^W, r_j^U) are known, we write this model as:

$$P\left(Obs_j^W,\, Obs_j^U, r_j^W,\, r_j^U,\, I_j \,|\theta\right) = P\left(I_j\,|\theta\right) P\left(r_j^W,\, r_j^U \,|\, I_j, \theta\right) P\left(Obs_j^W,\, Obs_j^U \,|r_j^W,\, r_j^U,\, \theta\right)$$

The first term of the right-hand size is the infection (i.e. virus circulation) model. The second term is the antibody response model, and the third term is the observation model. The subscript *j* is used to index the individuals. θ is the vector of parameters. I_j is the infection history which can take four values (0,0), (1,0), (0,1), (1,1), in which the first and second terms characterize whether individual *j* was infected by WNV and USUV, respectively.

The observation model

The observation model characterizes how the observed titers are distributed with respect to the real (unobserved latent) titer. The 'real' titers for individual *j* for WNV and USUV are noted r_j^W and r_j^U, respectively. We make several assumptions. First, observed values are at most one titer unit apart from the real value. Therefore, if the real titer is *r*, the observed value can be *r*-1, *r*, or *r*+1. We distinguish the parameters characterizing the occurrence of false negatives (ε_{fn}) and false positives (ε_{fp}) from other types of errors ($\frac{\varepsilon}{2}$). Two replicates of the observed values are available for each individual which may differ from the real titers. We consider that the observations are independent so that:

$$P\left(Obs_j^W,\, Obs_j^U \,|r_j^W,\, r_j^U,\, \theta\right) = P\left(o_1^W \,|r_j^W,\, \theta\right).P\left(o_2^W \,|r_j^W,\, \theta\right).P\left(o_1^U \,|r_j^U,\, \theta\right).P\left(o_2^W \,|r_j^U,\, \theta\right)$$

These terms depend on the six error terms ε^W, ε_{FP}^W, ε_{FN}^W, ε^U, ε_{FP}^U, ε_{FN}^U and are detailed in Table 1. By assuming that the observations are at most one titer value away from the real value, the replicates can be equal, different by one titer, or different by two titer values. For instance, if the real value is 2 (*r* = 2), the observed pair of titers can be either (1,1), (1,2), (2,1), (2,2), (3,2), (2,3), (3,1) or (1,3). Conversely, if for instance the observed titers are (1,2), the possible real value is 1 or 2, but not 3 because 1 and 3 are two titers apart. Moreover, we account for right-censoring for real titers larger than 4. In the conditional observation probability, we write $P(4|r,\theta) = 1$.

Table 1. Conditional probability of observing a titer value for all combinations included in the observation model. The probabilities that are not mentioned are equal to 0.

Observed (o)	Real (r)	Probability ($P(o\|r)$)
0	0	$1-\varepsilon_{FP}$
1	0	ε_{FP}
0	1	ε_{FN}
1	1	$1-\varepsilon_{FN}-\frac{\varepsilon}{2}$
2	1	$\frac{\varepsilon}{2}$
1	2	$\frac{\varepsilon}{2}$
2	2	$1-\varepsilon$
3	2	$\frac{\varepsilon}{2}$
2	3	$\frac{\varepsilon}{2}$
3	3	$1-\varepsilon$
4	3	$\frac{\varepsilon}{2}$
3	4	$\frac{\varepsilon}{2}$
4	4	$1-\frac{\varepsilon}{2}$
4	>4	1

The infection model

The first term in the hierarchical model, $P(I_j \,|\theta)$, is the probability that among the ELISA positive, an individual had a specific history of infection by WNV and USUV. Assuming that the probability of infection by both viruses are independent, at each period, the relation between the probability of infection and infection status is given by the set of four equations:

Not previously infected: $P_t(I = (0,0)|\,\theta) = (1-P_t^W).\,(1-P_t^U)$
Previously WNV infected: $P_t(I = (1,0)|\,\theta) = P_t^W.\,(1-P_t^U)$
Previously USUV infected: $P_t(I = (0,1)|\,\theta) = (1-P_t^W).P_t^U$
Previously WNV and USUV infected: $P_t(I = (1,1)|\,\theta) = P_t^W.P_t^U$

The antibody response model

The antibody response model characterizes the distribution of the antibody response for both viruses provided that the infection history is known. We assume antibody responses are due to infection and not maternal antibodies and that neutralizing titers arising from the infection with a virus (and cross-reactivity) follow a zero-truncated Poisson distribution.

The model extends the one developed in Hozé et al. (183) and we recall the general line here. If individual *j* has not been infected by either WNV or USUV, they have no response and the probability of having titers r_j^W and r_j^U is $P\left(r_j^W, r_j^U \middle| I_j = (0,0), \theta\right) = 1$ and in case r_j^W and/or $r_j^U \neq 0$, $P\left(r_j^W, r_j^U \middle| I_j = (0,0), \theta\right) = 0$. In case of the other infection history options, i.e., $I_j = (1,0)$, $I_j = (0,1)$ and $I_j = (1,1)$, the model follows as described in Hozé et al. (183) and includes the direct and cross-reactive response due to infection given by a zero-truncated Poisson distribution. In the case of an infection by WNV and not by USUV, the direct response is indicated by the parameter σ^W. A cross-reactive USUV response occurs with a probability $p^{W\to U}$, and the titer increase is given by the parameter $\sigma^{W\to U}$. Moreover, we assume that the cross-reactive response does not exceed the direct response and introduce a parameter $f^{W\to U} \leq 1$ such that $\sigma^{W\to U} = f^{W\to U} \times \sigma^W$. In the case of an infection with both viruses, both the direct and the cross-reactive responses contribute to the titer increase.

The likelihood

The model is obtained by summing over all the possible real titer values and all infection histories for individual *j* which can be summarized as:

$$P_j = \sum_{I_j} \sum_{r_j^W} \sum_{r_j^U} P\left(I_j \middle| \theta\right) P\left(r_j^W, r_j^U \middle| I_j, \theta\right) P\left(Obs_j^W, Obs_j^U \middle| r_j^W, r_j^U, \theta\right)$$

The loglikelihood is obtained over all individuals and is given by:

$$LL = \sum_j \log P_j$$

In addition, negative ELISA assays contribute to the estimation of the error terms (ε_{FP}^{U} and ε_{FP}^{W}). We write E_0 for the total number of negative ELISA assays, E_0^W (respectively E_0^U) for the total number of negative ELISA boars that are positive for WNV in VNT (respectively, USUV) and we assume a Binomial distribution for the number of negative ELISA results. We assume a perfect sensitivity of the ELISA (130) which means that ELISA negative samples with a positive VNT result are considered false positives. ELISA assays' contribution to the loglikelihood is:

$$LL = \log P\left(E_0^W; E_0, \varepsilon_{FP}^W\right) + \log P\left(E_0^U; E_0, \varepsilon_{FP}^U\right)$$
$$= E_0^W \log \varepsilon_{FP}^W + (E_0 - E_0^W)\log(1 - \varepsilon_{FP}^W) + E_0^U \log \varepsilon_{FP}^U + (E_0 - E_0^U)\log(1 - \varepsilon_{FP}^U)$$

Estimation of infection probability from ELISA assays

For each time period (*t*), we estimate the infection probability from ELISA assays for WNV and USUV (π_t^W, π_t^U), from the estimated posterior probability of positive VNTs (P_t^I) among the ELISA positive samples. The ELISA positive tests arise from a binomial process, such that

$$Pos_t \sim Binomial\left(E_{total,t}, \frac{E_{pos,t}}{E_{total,t}}\right),$$

where $E_{total,t}$ is the total ELISA tests observed and $E_{pos,t}$ is the number of positive ELISA tests observed for either WNV or USUV in period *t*. The overall probability of infection is then given by:

$$\pi_t^I = P_t^I \cdot \frac{Pos_t}{E_{total,t}}.$$

Prediction calculation

We use the model to predict the observed joint response in the case of an infection. When the infection status is known, the probability of observing a set of titers is obtained by accounting for the possible real titers that generated the observation:

$$P\left(Obs_j^W, Obs_j^U \middle| I_j, \theta\right) = \sum_{r_j^W, r_j^U} \left(Obs_j^W, Obs_j^U \middle| r_j^W, r_j^U, I_j, \theta\right) P\left(r_j^W, r_j^U \middle| I_j, \theta\right)$$

Parameter estimation

The priors were set as non-informative for all parameters used in the model. For the cross-reaction probabilities ($p^{W\to U}$ and $p^{U\to W}$) and observation errors (ε^W, ε_{FP}^W, ε_{FN}^W, ε^U, ε_{FP}^U, ε_{FN}^U), we used uniform distributions ~Uniform (0, 1). We introduce the terms for the prevalence within the ELISA positive population λ_t^W and λ_t^U that are implemented in the infection model as follows: $P_t^I = 1 - e^{-\lambda_t^I}$ and followed uniform distributions ~Uniform(0, 10), and the titers ($\sigma^W, \sigma^U, \sigma^{W\to U}, and\ \sigma^{U\to W}$) ~Uniform (0, 4). The reduction parameters $f^{W\to U}$ and $f^{U\to W}$ follow a Uniform(0, 1) distribution. We fitted the model parameters using a Markov

chain Monte-Carlo (MCMC) framework implemented in the rstan package. A No U-Turn sampler variant of Hamiltonian Monte-Carlo was used to update the parameters. Two chains of 15000 iterations with a burnin of 5000 iterations were ran, thinning each 3 samples. The analyses were run in R Core Team (2023). Model diagnostics were assessed with the trace plot, Gelman Rubin plots, and potential scale reduction factors convergence diagnostic. The diagnostics show that all models converged properly.

Sensitivity analysis

We set three scenarios to test the sensitivity of the model results. First, we decreased the VNT cut-off compared to the baseline model for WNV to 10 (same as USUV), which is called model 1. Furthermore, we tested a model in which $p^{W \to U}$ and $p^{U \to W}$ are set to zero, using the cut-off titer of 30 for WNV and 10 for USUV (model 2). Later, we tested a model in which $p^{W \to U}$ and $p^{U \to W}$ are set to zero but using the cut-off 10 for both WNV and USUV (model 3). The reason for using the different WNV cut-offs is that an initial cut-off of 10 was used for WNV, but information from ELISA negative Dutch boars and the set of Norwegian boars informed us about the more appropriate cut-off of 30. All simulations followed the same settings explained in the parameter estimation section. Model comparison was performed using the PSIS-LOO criterion which relies on computing a Bayesian leave-one-out estimate of the log pointwise predictive density (LPPD), and to which a standard error is associated. The loo_compare function in the loo package compares the expected log pointwise predictive density (ELPD) and its associated standard error (SE) for the different models and was used for model selection (189,190).

Model adequacy

Firstly, we assessed the model adequacy by simulating 1000 surveys in a population with the same characteristics as the data (sample size and period). These simulations were compared to the data based on the frequency of observing specific titers and the frequency of observing concordant samples. We compare the model with the data based on 1) the frequency with which titers were observed and 2) the frequency of concordant samples. After, we used the median estimates of the model's output (baseline and the sensitivity analysis) to generate three datasets. Then, we fit the model using the in-silico data and assessed whether the outputs contain the parameters within the 95% credible intervals.

3. Results

3.1. Descriptive results and sample size

A total of 1,504 wild boar sera, collected between January 1st 2018 and December 31st 2021, were randomly selected from the sample database. These comprised of 41.69%

females, 44.74% males and 13.56% boars with unreported sex. The majority (58.64%) of animals were 1-12 months of age, 29.19% were 13-24 months and 7.65% were over 24 months. For the remaining 4.52% the age was unknown. The mean (estimated) age was 13.57 months, with an interquartile range of 6 to 18 months. Table 2 shows the age, sex, and sampling province for the boar included in the study per year.

Table 2. Descriptive results of wild boars (*Sus scrofa*) included in the study per year. *[1] Mean (IQR); [2] n (%).*In 2018 and 2021, limited/no sera were collected in the province of Limburg, due to shortage in personnel.*

Characteristic	Year			
	2018, N = 372	2019, N = 390	2020, N = 354	2021, N = 388
Age in months	13 (6, 18)[1]	13 (5, 18)[1]	14 (6, 18)[1]	14 (8, 18)[1]
Unknown	23	2	30	13
Age group				
1-12 months	214 (61%)[2]	241 (62%)[2]	201 (62%)[2]	226 (60%)[2]
13-24 months	107 (31%)	118 (30%)	94 (29%)	120 (32%)
25-36 months	24 (6.9%)	22 (5.7%)	17 (5.2%)	19 (5.1%)
>36 months	4 (1.1%)	7 (1.8%)	12 (3.7%)	10 (2.7%)
Unknown	23	2	30	13
Sex				
Female	113 (50%)	167 (45%)	170 (52%)	177 (47%)
Male	113 (50%)	203 (55%)	160 (48%)	197 (53%)
Unknown	146	20	24	14
Province*				
Drenthe	0 (0%)	1 (0.3%)	1 (0.3%)	0 (0%)
Gelderland	4 (2.8%)	3 (0.8%)	16 (4.6%)	0 (0%)
Limburg	2 (1.4%)	211 (55%)	114 (33%)	0 (0%)
Noord-Brabant	137 (94%)	156 (41%)	199 (58%)	387 (100%)
Overijssel	2 (1.4%)	11 (2.9%)	15 (4.3%)	0 (0%)
Unknown	227	8	9	1

In total, 30.65% (461/1,504) of sera had a doubtful or positive ELISA result. A declining trend in ELISA positives was observed over time; from 44.89% (167/372) to 31.54% (123/390), 25.71% (91/354), and 20.62% (80/388) per year for 2018 to 2021, respectively. Five ELISA positive samples, three from 2018, one from 2019 and one from 2021, could not be tested in VNTs due to insufficient remaining volume. VNTs could not be interpreted (titer <90 or <270) in 5, 7, 31, and 8 samples from 2018, 2019, 2020, and 2021, respectively. These fifty-six ELISA positive sera (12.15%, 56/461) had to be excluded from further analyses. Thus, 405 ELISA positive individuals were

included in the statistical model. Of these, 159, 115, 60, and 71 were from 2018-2021, respectively. A summary of the serosurvey results using a classical interpretation approach (four-fold titer difference between two viruses) is shown in Table 3.

Table 3. Results of the 1,504 wild boar serum samples from 2018 up to 2021 in the Netherlands based on a four-fold VNT titer difference. Titers of <10 and <30 are regarded negative. Bad serum quality includes all ELISA positive boars with VNT values of <90 or <270 or not able to be tested in VNT at all. [1] *(% (n))*

Result	**2018**	**2019**	**2020**	**2021**
USUV	7.80% (29)[1]	1.28% (5)	1.98% (7)	0.52% (2)
WNV	6.99% (26)	9.23% (36)	3.39% (12)	4.38% (17)
Equivocal	21.24% (79)	11.03% (43)	5.08% (18)	8.25% (32)
Bad serum quality	2.15% (8)	2.05% (8)	8.76% (31)	2.32% (9)
Negative	61.83% (230)	76.41% (298)	80.79% (286)	84.54% (328)
Total	372	390	354	388

3.2. Model results

The expected neutralizing titer following infection with WNV was 39.97 (33.49-48.42, 95% CrI), and 42.41 (30.83-58.45, 95% CrI) for USUV. Infection with WNV increased USUV titers with 27.21 (15.23-40.01, 95% CrI), while infection with USUV increased WNV titers with 31.87 (30.07-37.79, 95% CrI). These titer values were obtained by transforming the posterior densities observed in Figure 1A according $10 \cdot 3^{\sigma-1}$, for USUV and $10 \cdot 3^{\sigma}$ for WNV. The probability of a cross-reactive (USUV) response after WNV infection was 53.0% (26.6--69.7%), while the probability of a cross-reactive (WNV) response after USUV infection was higher, namely 63.2% (38.4-77.2%) (Figure 1B). The probability of false positives (ε_{FP}) was higher for WNV (9.1% [5.7-13.0%, 95% CrI]) than for USUV (2.0% [1.1-3.8%, 95% CrI]) as the credible intervals do not overlap (Figure 1C). The probability of false negatives (ε_{FN}) was 10.4% for WNV (5.6-15.9%, 95% CrI) and 3.0% for USUV (1.1-9.5%, 95% CrI). The probabilities of other observational errors (ε) were 23.2% (17.7-30.5%, 95% CrI) for WNV and 25.9% (20.9-32.0%, 95% CrI) for USUV. The λ_t^I posterior distributions show a declining trend for USUV positives and increasing trend for WNV positives among the ELISA positive wild boars. Especially in 2018, the USUV posterior distribution was higher than for WNV (Figure 1D).

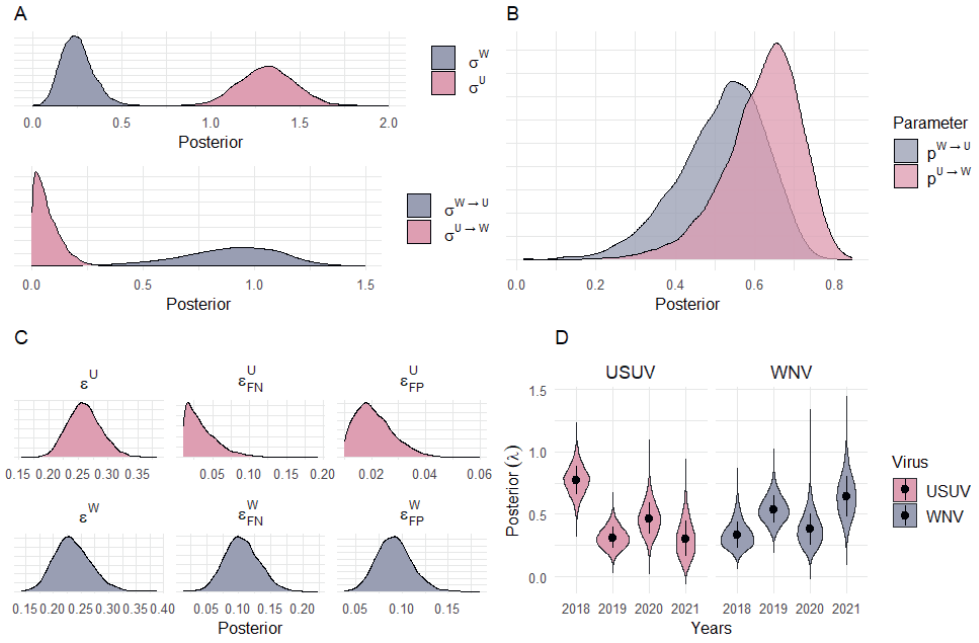


Figure 1. Posterior density distributions for the WNV and USUV titers (A), cross-reaction probability between WNV and USUV (B), observation errors (C), and λ_t^I for WNV and USUV (D).

3.3. Probability of infection prior to sampling

The probabilities of infection prior to sampling of WNV from 2018 to 2021 were 12.4% (6.9-20.0%, 95% CrI), 13.0% (9.0-17.5%, 95% CrI), 7.8% (3.9-12.9%, 95% CrI), and 9.6% (5.6-13.8%, 95%CrI), respectively. For USUV, the probabilities from 2018 to 2021 were 24.0% (18.4-29.4%, 95% CrI), 8.3% (4.6-12.4%, 95% CrI), 9.4% (5.3-14.0%, 95% CrI), and 5.2% (1.4-9.8%, 95% CrI) respectively (Figure 2). We thus observe similar trends compared to using the classical interpretation approach. However, the actual estimated probabilities are higher for both viruses and all years in the model compared to the classical interpretation, especially for USUV in 2018.

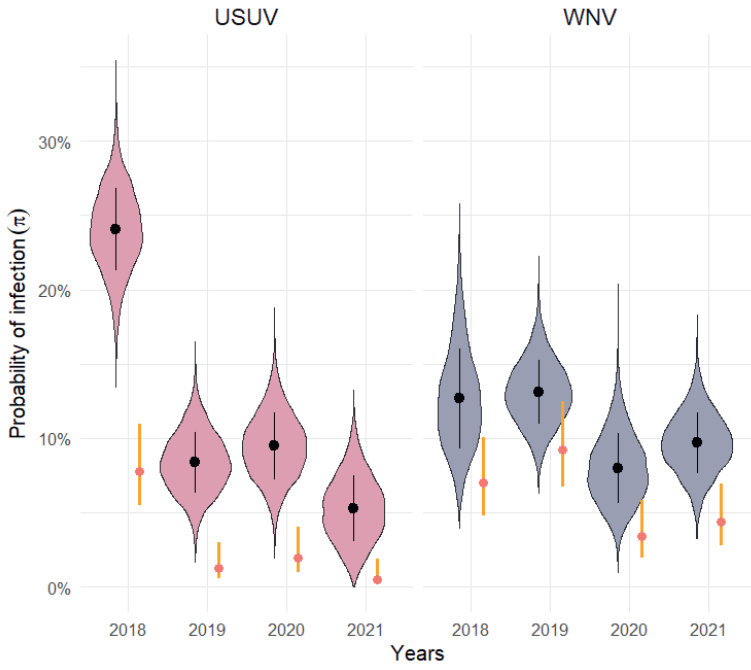


Figure 2. Probability of infection (π) in or before year (*t*) of WNV and USUV in wild boars from 2018 to 2021 in the Netherlands. The orange ranges are the observed prevalence and 95% confidence interval (calculated according Wilson method) based on the classical interpretation (four-fold titer difference, as shown in Table 3).

3.4. Prediction calculation

Next, we examined how our model could be used to evaluate the accuracy of serological diagnostic of WNV and USUV infection. We generated titer distributions conditioned on hypothetical known infection status for individuals infected with only USUV (Figure 3A), only WNV (Figure 3B), or co-infection (Figure 3C). The model predicts that USUV infection will lead to an increase in WNV titer in 63% of the instances, where WNV infection will result in USUV titer in 53% of the instances. Upon an USUV infection, 43% of individuals are found USUV positive with classical method of serological diagnostic, 6% are found WNV positive and 52% are equivocal. Upon a WNV infection, 7% are found USUV positive with classical method of serological diagnostic, 50% are found WNV positive and 39% are equivocal.

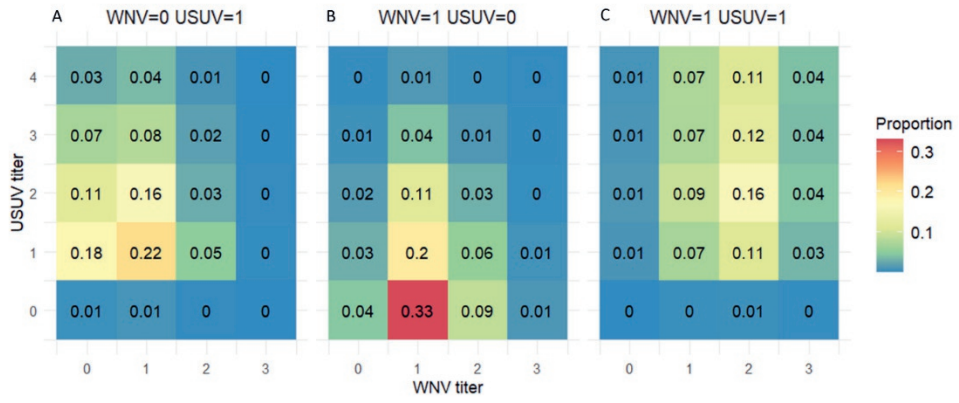


Figure 3. Proportion of animals with respective r_j^W and r_j^U (WNV on x-axis, USUV on y-axis) after single (panel A & B) or co-infection (panel C). USUV or WNV=1, means positive status for infection and USUV or WNV=0, means negative status for infection.

3.5. Sensitivity analysis

Model 1 (WNV and USUV cut-off at 10) results in higher median cross-reactivity titers from WNV to USUV ($\sigma^{W\to U}$), namely 0.43 instead of 0.05 in the baseline model. The median cross-reactivity titers from USUV to WNV ($\sigma^{U\to W}$) was lower in model 1 compared to baseline (0.32 instead of 0.91). The probability of cross-reaction from WNV to USUV ($p^{W\to U}$) decreased from 0.53 to 0.21, and the probability of cross-reaction from USUV to WNV ($p^{U\to W}$) showed a increse, from 0.63 to 0.81. As model 2 and 3 represent a scenarios without cross-reactivity, cross-reactivity titers and the probability of cross-reaction are not depicted in Figure 4.

For both model 1 and model 3, the VNT cut-off for WNV was reduced from 30 to 10. This results in an increase in the antibody response for WNV, but has limited effect on USUV antibody response besides increased titers for models without cross-reaction (Figure 4A). For the observational errors, all models lead to minor changes compared to the baseline (less than 3%) and the results were omitted from Figure 4. It is observed that the baseline model has lower λ_t^I in all years compared to the other scenarios for both diseases (Figure 4B).

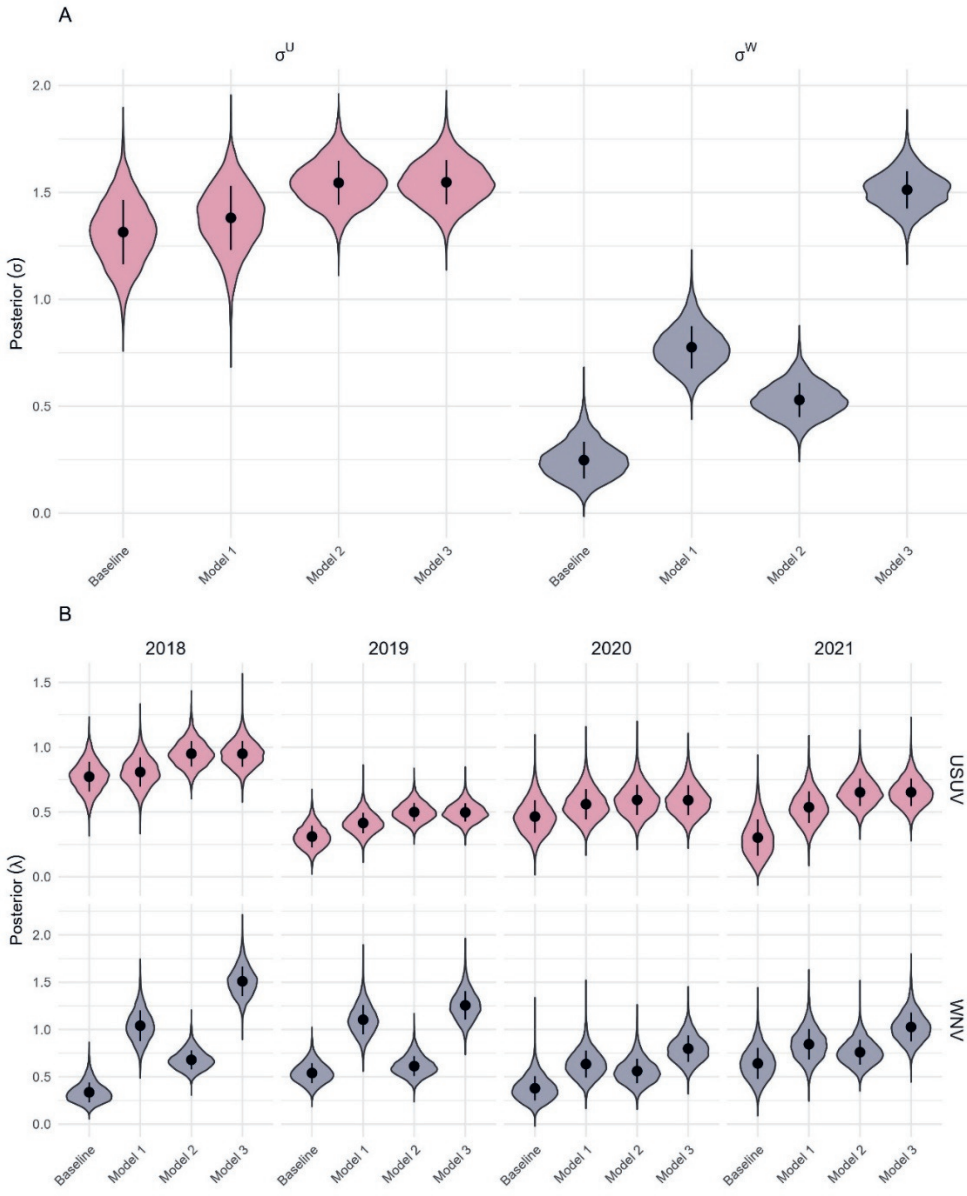


Figure 4. Posterior distributions for titers σ^W, σ^U (A), and λ^I over 2018, 2019, 2020, and 2021 (B). The values are the posterior for: model 1 (WNV and USUV cut-off at 10), model 2 (no cross-reaction, WNV and USUV cut-off at 10), and model 3 (no cross-reaction, cut-offs same as baseline). The observation errors ε^W, ε^U, ε^W_{FN}, ε^U_{FN}, and ε^W_{FP}, ε^U_{FP} showed minor changes compared to the baseline and were omitted.

3.6. Model adequacy

The model was able to retrieve most of the parameters used to generate the in-silico database. However, the 95% credible intervals for λ_2^U, λ_2^W, ε_{FN}^W, ε_{FP}^W and ε_{FN}^U are not included within the 95% credible interval. Results of the model adequacy are shown in Supplementary Material 1 (Figures S3-S5). The LOOICs of the models indicated that both in the baseline dataset and the dataset in which the cut-off was set at 10, the model that considers cross-reaction performed better (Table 4).

Table 4. Model fitness comparison using LOOIC in four different models used in the sensitivity analysis.

	Cross-reactivity	**No cross-reactivity**
USUV cut-off at 10, WNV cut-off at 30	3067.5* (Baseline)	3212.5 (Model 2)
USUV and WNV cut-off at 10	3526.2* (Model 1)	3661.8 (Model 3)

*best performing model

4. Discussion

With this study we provide epidemiological insights into USUV and WNV infections in wild boars in the Netherlands over the years 2018-2021. We found previously undetected circulation of WNV in, or before, 2018. We show the added value of using a mathematical and statistical approach to interpret serological results of cross-reacting and co-circulating viruses, which confirms our findings.

Firstly, the high proportion of wild boars with neutralizing antibody titers throughout our study period was surprising. This is in contrast to a serosurvey in equines and dogs in the Netherlands in 2021-2022 (166). Furthermore, we show evidence of (local) infections of WNV in animals before the first official detection of the virus in 2020 (58). Both the classical interpretation as well as the model results indicate a WNV probability of infection of at least 7%, thus providing robust evidence of WNV introduction(s) in or before 2018. In 2018, a large outbreak of WNV caused over 1,500 human cases in the European Union and the virus was first found in Germany (29). The warm summer of 2018 provided the optimal conditions for WNV circulation, which could also explain potential introduction(s) into the Netherlands (191). WNV seropositivity has been detected before 2017 in (migrating) wild birds, but virus detection was not reported until 2020 (58,192). Our findings could indicate that WNV was only introduced or spread locally, and no domestic animals or humans got infected. It is important to note that the presence of wild boars, and thus our samples, is geographically limited and therefore does not provide a comprehensive representation of the entire country. Nevertheless, even in 2018 when there were no samples from the southern-most province (Limburg) we found evidence for WNV

infections. For USUV we observe a declining trend with regards to the probability of infections, which could be due to the large outbreak in 2016 followed by lower circulation in the years after as was also observed in wild bird populations (55,192).

The model results reveal that we can expect a slightly higher direct titer response for USUV compared to WNV, while credible intervals are almost similar. However, the cross-reactive WNV response due to an USUV infection was higher indicating that the direct and cross-reactive WNV titers will be closer to each other compared to USUV titers. Furthermore, the probability of a cross-reactive WNV response after an USUV infection (63%) was higher than vice versa (53%). In the prediction calculation, this results in the fact that less USUV infected individuals are correctly identified as USUV infected. This finding is also reflected in the difference between the probability of infection estimation by the model compared to the classical interpretation. Here we observe a much higher probability of infection for USUV compared to WNV in 2018, while these probabilities are almost equal in the classical interpretation. The model is able to 'allocate' the equivocal individuals to either USUV, WNV, or both. The higher probability of USUV infection in 2018 is in accordance with the fact that the Netherlands experienced a large outbreak in 2016 and the virus had been detected, in a declining trend, in the years after (55,56,192).

The sensitivity analysis highlights the need for accounting the cross-reaction (Table 4). It reinforces that the model deepens the understanding of the different processes that lead to the observations in the population. Our results show that the probability of cross-reactivity is highly dependent on the cut-off chosen for the VNT. However, establishing cut-offs in diagnostic tests is a complex task that requires targeted studies to ensure diagnostic validation and accuracy. In literature, multiple approaches have been described to establish correct cut-offs. One could argue that ideally, populations with known infection status should be used to validate the model and thereby interpret serological results. Unfortunately, these samples were not available for Dutch wild boars as samples from before 2018 do not exist. USUV (55) and potentially WNV (as indicated by our results) were already present in 2018. Another approach is the use of latent class models, which have been employed for over 20 years to address situations where a perfect reference (gold standard) is unavailable, allowing tests to be evaluated against an imperfect reference. Since 2017, the STARD-BLCM standard has been available for reporting diagnostic accuracy studies in veterinary medicine using Bayesian latent class models (193). As a limitation of this study, we set up the cut-offs based on positive reference samples from horses and bird species for both WNV and USUV. For future research, we recommend to further study the optimum cut-offs using more appropriate positive and negative reference samples, as well as development of (more) specific diagnostic assays.

The model adequacy showed us that the model was able to retrieve most of the parameters correctly. However, for λ_2^W, λ_2^U, ε_{FN}^W, ε_{FP}^U, and ε_{FP}^W this was not the case. The 95% credible interval obtained by fitting the data was higher than the in-silico parameter for λ_2^U but lower for λ_2^W. This could indicate that the model underestimated the probability of infection for USUV in 2019, while it overestimated the probability of infection for WNV. This is plausible when looking at Figure 1D and Figure 2, where we observe an off-trend distribution for 2019 for both viruses. This could potentially be explained by more persistent WNV antibodies (slower waning) compared to USUV, resulting in classification of historical USUV infections as WNV infections. However, very limited evidence exists for the duration of persistence of these antibodies, let alone in wild boars with sequential or co-infections (102,194,195).

Their potentially high exposure to vectors, limited home range, as well as the fact that they are not vaccinated makes wild boars suitable for surveillance of orthoflaviviruses. In the Netherlands, wild boar samples are collected each year for other surveillance purposes (196). Disadvantages of the use of wild boars are poor serum sample quality and the limited geographical range of wild boars in the Netherlands. Surveillance of wild boars could be supplemented by increasing sampling efforts of other (dead-end) hosts such as wild birds, equines or chickens around the seropositive wild boars to further investigate local circulation of USUV and WNV. Furthermore, it is of utmost importance to preserve sets of negative and positive control sera to be able to interpret serological results in the future. Despite these limitations, this study shows the added value of using a mathematical and statistical approach to interpret orthoflavivirus serology as well as the potential of using wild boars in orthoflavivirus surveillance.

Acknowledgements

The authors would like to thank Michiel Kroese for his assistance with sample selection and contact with the appropriate authorities. Bregtje Smid is acknowledged for performing Virus Neutralization Tests on wild boar and pig samples. We also would like to thank the Ministry of Agriculture, Fisheries, Food Security and Nature for their permission to use the samples included in this study.

Supplementary material

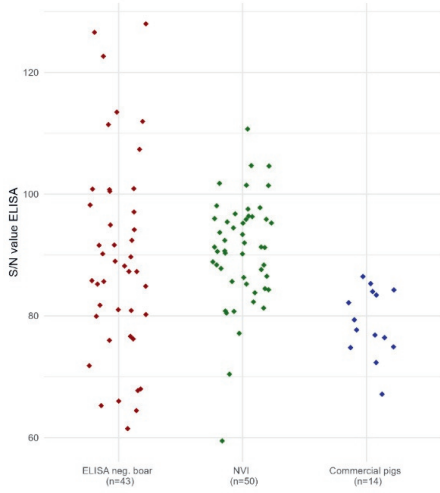


Figure S1. ELISA results of the negative control groups (ELISA neg. boar: random selection of wild boars from our dataset that were ELISA negative, NVI= Norwegian wild boars sampled in 2018-2019, commercial pigs = Dutch slaughterhouse samples from commercial pigs). S/N% values <50 are considered positive according to the manufacturer's instructions.

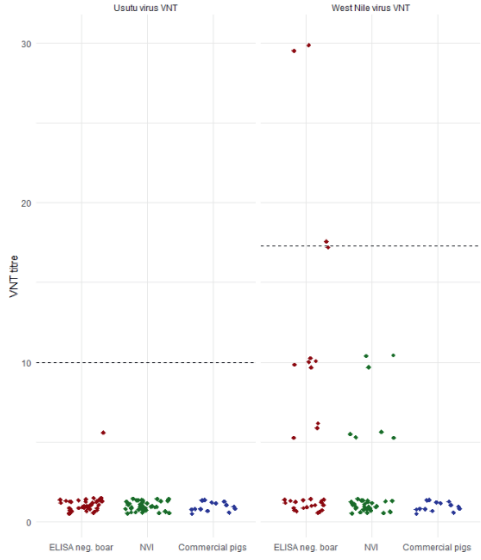


Figure S2. VNT results of the negative control groups for Usutu and West Nile virus, dashed lines indicate cut-offs for both VNTs (ELISA neg. boar: random selection of wild boar from our data that were ELISA negative, NVI= Norwegian wild boars sampled in 2018-2019, commercial pigs = Dutch slaughterhouse samples from commercial pigs).

Model adequacy

Figure S3. Comparison of the model predicted titer responses (model) and the observed titer response (data) for Usutu virus (USUV) and West Nile virus (WNV).

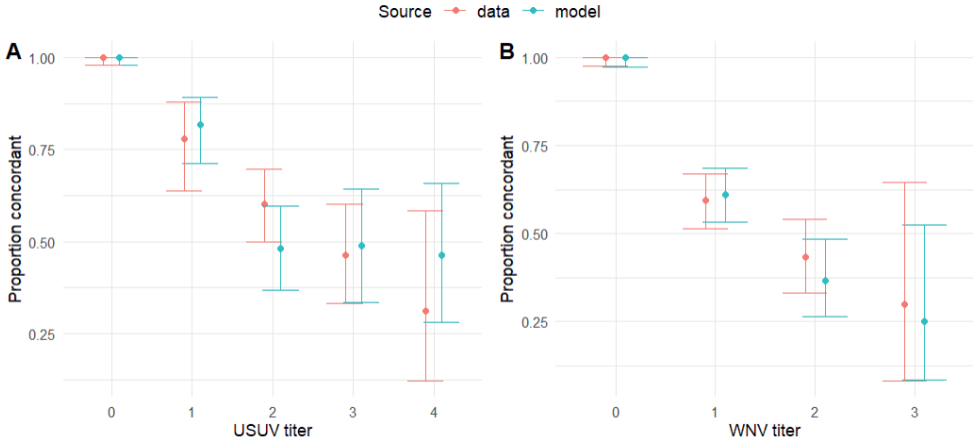


Figure S4. Proportion concordant and 95% credible interval of replicates for USUV (A) and WNV (B).

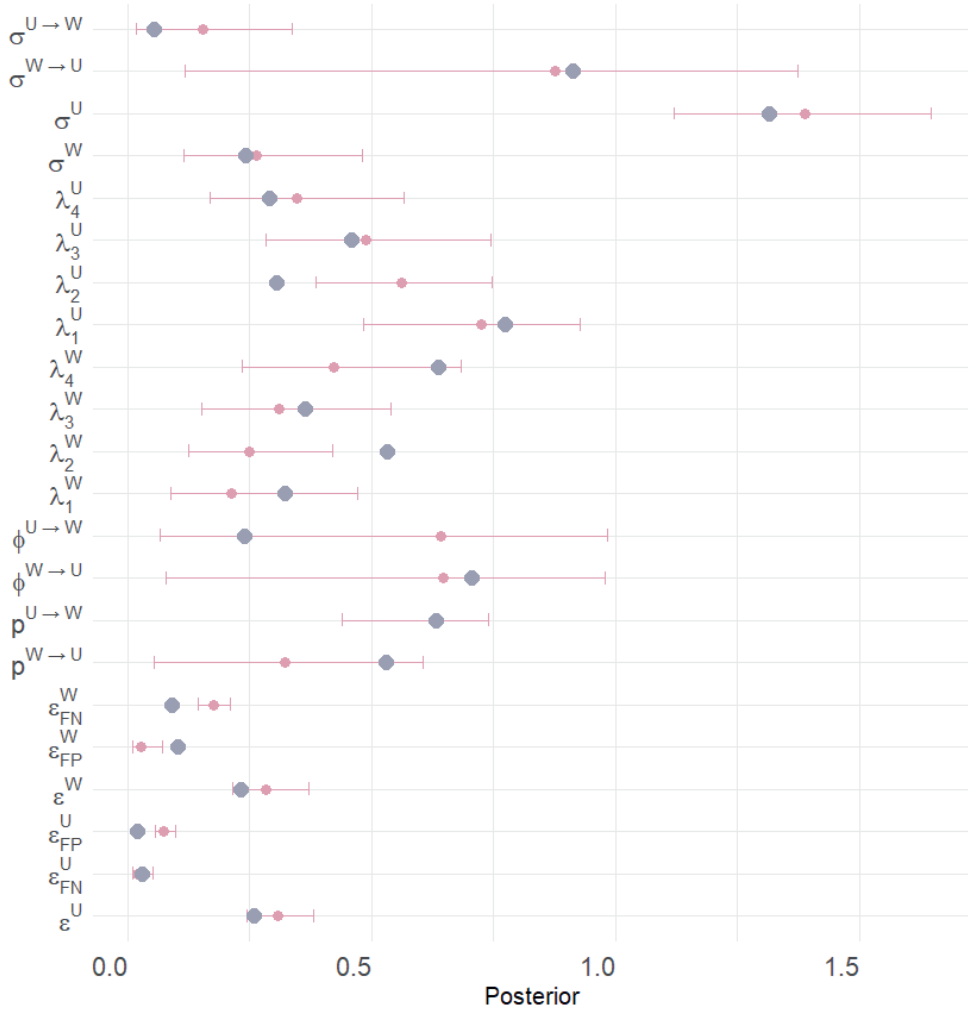


Figure S5. Posterior distribution (median and 95% credible interval) of the model's parameters obtained by fitting the baseline model to the in-silico data. The gray dots are the in-silico parameters used to generate the dataset and the pink interval is the 95% credible interval obtained by fitting the data.

Chapter 5.

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

Kiki Streng*[1], Nnomzie Atama*[2], Felicity Chandler[2], Rody Blom[3], Henk van der Jeugd[4], Maarten Schrama[5], Marion P. G. Koopmans[2], Wim H. M. van der Poel[1,6], Reina S. Sikkema[2]

* Authors contributed equally

Published in Emerging Microbes & Infections (2024)

Affiliations
[1] Quantitative Veterinary Epidemiology, Wageningen University and Research, Wageningen, the Netherlands
[2] Viroscience, ErasmusMC, Rotterdam, the Netherlands
[3] Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands
[4] Dutch Centre for Avian Migration and Demography, NIOO – KNAW, Wageningen, the Netherlands
[5] Institute of Environmental Sciences, Leiden University, Leiden, the Netherlands
[6] 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 analyzed 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 behavior.

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.

1. 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* (197). 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 (180,198,199). *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 (35,200,201). *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 (202).

Usutu virus has been circulating in continental Europe for more than two decades (203). 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 (55,56). 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 (123). Surveillance of live and dead wild birds and mosquitoes has shown ongoing circulation of USUV in the years after (192).

Over the last decades, WNV has become one of the most widespread arboviruses in the world (204). 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 (29). In the same year, the virus also was detected northwards as the first WNV cases in Germany were observed in birds and horses (205). In August 2020, the virus was detected for the first time in the Netherlands in a common whitethroat (*Curruca communis*) and *Culex* mosquitoes (58). 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 (207). 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 (125,207).

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 (5). 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 (76,208,209). 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 (210,211). 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. Sampling of sentinel chickens allows for repeated sampling of the same individuals. 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 (212). Of the about 500 petting zoos in the country, 90% keep chickens alongside other animals, such as peacocks, sheep and goats (212). 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 (213).

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.

2. Materials and methods

2.1. Sampling

Chickens from petting zoos and backyards within a 15km radius of each of the two WNV outbreak locations (58,125,207) 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 minutes to collect sera, which were stored at -80°C until use.

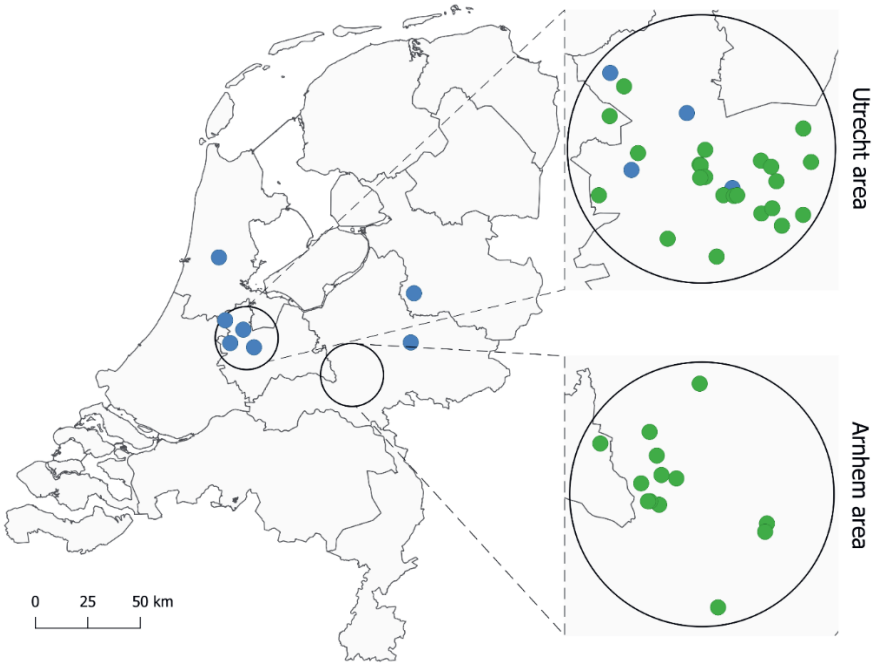


Figure 1. Sampling locations (green dots) within the 15km radius of two locations where WNV was detected in 2020. Locations where backyard chickens were sampled by volunteer bird ringers are shown in the full country map and the Utrecht area inset as blue dots.

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 50mL falcon tubes at -20°C until identification and further processing at the laboratory.

2.2. Laboratory analysis

2.2.1. Protein microarray

All sera with sufficient volume were tested on a protein microarray (PMA) as previously described (214). 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 ImmunoResearch, Inc., West Grove, USA). Each serum was tested in a 4-fold serial dilution ranging from 1:20 to 1:1280. The tested microarray slides were scanned using a PowerScanner™ (Tecan, Männedorf, Switzerland), and the relative fluorescence units (RFU) per antigen were analyzed using ImaGene® software (Biodiscovery, El Segundo, USA). Titres were calculated from the average RFU values using GraphPad Prism vs 9.4.0, as previously described (215).

Protein microarray cut-offs for chickens were calculated using a ROC curve, using antibody titres 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 (Supplementary material, Figure 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.

2.2.2. 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 (147). The positivity cut-off specific to chickens was established at ≥160 for USUV and ≥80 for WNV (Supplementary material, Figures S2 and S3). Samples showing positivity for both USUV and WNV mFRNTs, with a <4-fold titre difference between the two antigens are denoted as “FLAVI” i.e. orthoflavivirus-positive.

2.2.3 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 previously described (216). Viral cytopathic effects (CPE) were recorded five days post-inoculation for USUV and seven days post-inoculation for WNV. Sera were regarded positive in case of a reciprocal titre of ≥1:16 and a ≥4-fold titre difference was used to distinguish between WNV and USUV infections.

2.3. 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 titres across phases using a mixed-effect linear model using the lme4 package in RStudio (217). The dependent variable was the log-transformed mFRNT titre, 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 titre 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 (218).

2.4. Mosquito and bloodmeal host identification

Mosquitoes were identified to the species level following the identification key of Becker et al. (173). 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. (219). 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 analyzed 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.

3. Results

3.1. 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 15km radius of the two 2020 WNV outbreak locations in the Netherlands (see Table 1). Two samples from 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 15km radius in Utrecht (see Figure 1). Twenty-four samples (19 from 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).

3.2 Serology

3.2.1. 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% [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 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 Arnhem area in phase II, hence the seroprevalence was not estimated (see Table 2; Figure 2).

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

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)		

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

	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)
Arnhem	64.6 (62/96)	**WNV**	1.61 (1/62)	NA	**WNV**	NA	53.3 (40/75)	**WNV**	12.5 (5/40)
		USUV	32.4 (20/62) ǂ1		**USUV**	NA		**USUV**	15 (6/40)
		FLAVI	4.8 (3/62)		**FLAVI**	NA		**FLAVI**	2.5 (1/40)
Utrecht	42.3 (82/194)	**WNV**	8.8 (6/68)	59.9 (88/147)	**WNV**	22.6 (19/84)	55.2 (69/125)	**WNV**	22.7 (15/66)
		USUV	17.6 (12/68)		**USUV**	17.9 (15/84)		**USUV**	39.4 (26/66)
		FLAVI	10.3 (7/68)		**FLAVI**	8.3 (7/84)		**FLAVI**	9.1 (6/66)
Ringer locations	65.0 (13/20)	**WNV**	30.8 (4/13) ǂ4	100 (4/4)	**WNV**	25 (1/4)	100 (7/7)	**WNV**	14.3 (1/7)
		USUV	15.4 (2/13) ǂ2		**USUV**	0 (0/4)		**USUV**	14.3 (1/7)
		FLAVI	7.7 (1/13) ǂ1		**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)

Three out of seven volunteer bird ringer locations were located outside the two investigated areas. In phase I, in a location 40km north-west of Utrecht, one of two chickens tested WNV positive by VNT. In another location 22km 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 15km radius of the Utrecht area (see Figure 2A).

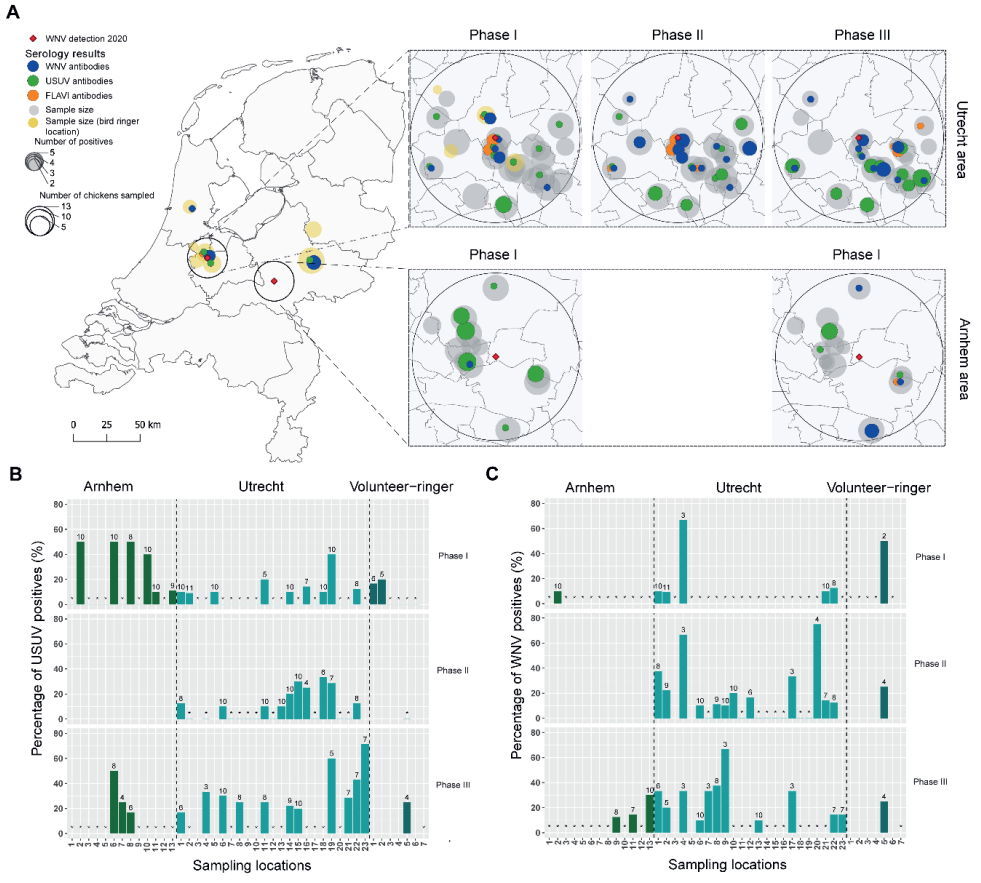


Figure 2. A: Spatial overview of all serological results for Utrecht and Arnhem area per sampling phase. Results for volunteer bird ringer locations outside of the two sampling areas (n=3), aggregated for all phases, are shown in the large left (country) panel. B: Percentage of USUV-positive chickens 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.

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 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.

3.2.2. 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.

3.3. 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 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 titre 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 titre of 432.7 for USUV at the first positive sampling. The model revealed an estimated average decline in log-transformed USUV titre of 0.049 per week since the first USUV status sample. This means the mFRNT titre of chickens would fall below cut-off (titre of 160) in about 20 weeks and below a titre of 10 (undetectable titre) 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 titres above cut-off (80, 80, 1280 and 2560 respectively) at all sampling points, thus no waning of antibody titres, over a period ranging from 115 and 439 days between first and last sampling. Seven chickens had a ≥4-fold decline in WNV titre (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.

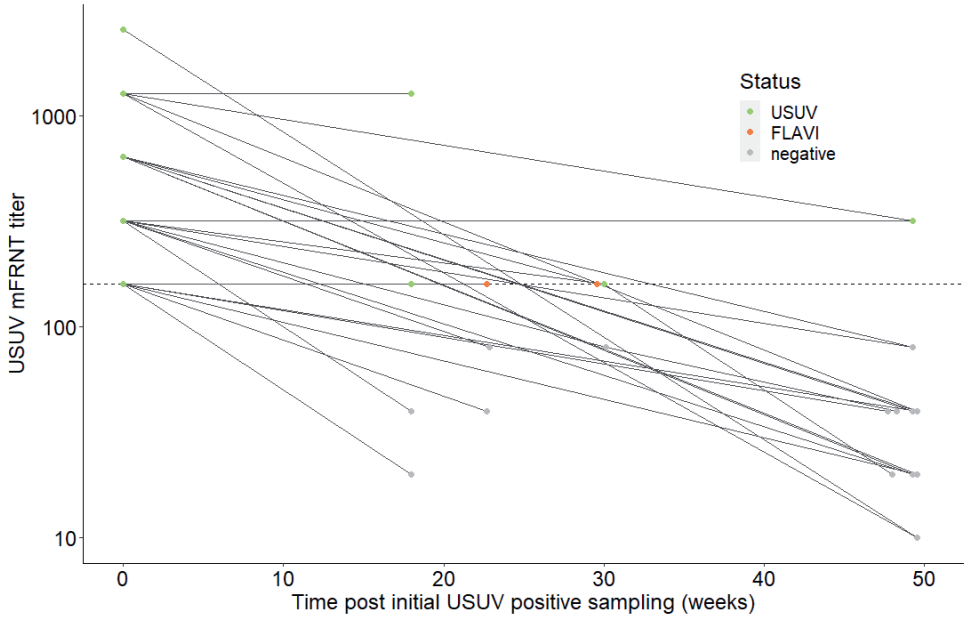


Figure 3. USUV mFRNT titres for 27 repeatedly sampled chickens confirmed positive for USUV in phase I or phase II. The horizontal dashed line indicates mFRNT cut-off titre for USUV. Status at each sampling point is indicated by a coloured dot.

3.4. 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 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.

4. 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 corroborated 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 (58). 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 (126). 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 (192). Surprisingly, no notable increase in blackbird mortality was observed in 2021 (220). Our findings underscore the significance of integrating chicken serological surveillance into existing 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 (208). Experimental infection studies often do not study long-term antibody kinetics (210). Our study estimates an average antibody decay to below cutoff at approximately 20 weeks post-initial positive sample. However, individual variation in neutralizing antibody development and persistence exists (76). Our findings align with a study in captive birds of prey showing a marked USUV antibody decline over six months (221). However, Bergmann et al. (194) 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 titres beyond 439 post-initial sampling. Compared to USUV, indeed longer WNV antibody persistence was previously reported in various bird species, (102,195,222,223). However, in none of these studied chickens were investigated.

A few seropositive chickens showed increasing titres over time, possibly due to new exposures or co-infections with other orthoflaviviruses (224). These factors are not easily discernible in field investigations. Some chickens maintained persistent high antibody titres 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 (225,226). 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 cutoff.

Apart from chickens, horses, dogs and other wildlife species are commonly used as sentinels for orthoflavivirus surveillance (95,97,185). However, horses may be vaccinated against WNV, rendering these individuals unsuitable as sentinels. Obtaining wildlife samples is challenging, and repeated sampling is often impractical (185). 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 (87). 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 (199,227). Surveillance programs 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.

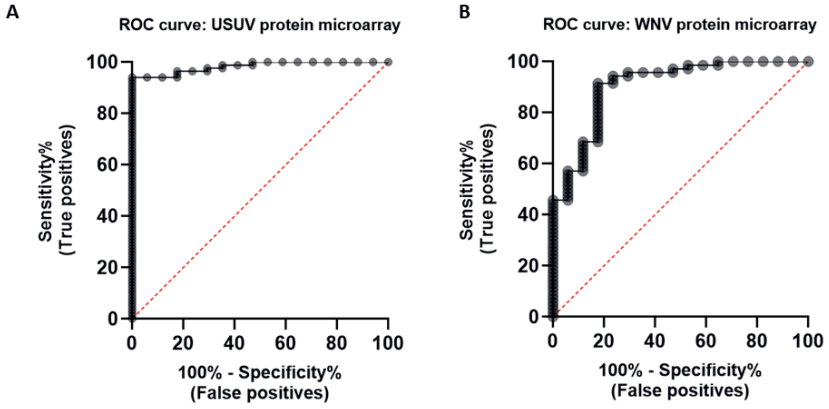
Overall, our study underscores the value of sentinel surveillance in petting zoos and backyard chickens in detecting virus circulation that might otherwise go unnoticed. Additionally, it affirms the utility of chickens as sentinels, complementing other surveillance methods such as wild bird and mosquito 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.

Acknowledgements

The authors would like to thank all petting zoos and chicken owners for participation in this study as well as Pauline de Best, Mariken de Wit, Chiara de Bellegarde, Laura Smeele, Christiaan Roos, and Jurrian van Irsel for their help with the sample collection. We thank Tijs van den Berg for logistical support and sample collection. Additionally, we thank Anne van der Linden and Mikaela Suehely Cicilia for their lab assistance, and Sjaak de Wit of Royal GD for providing the preARBO-TN samples used in the mFRNT and PMA validation.

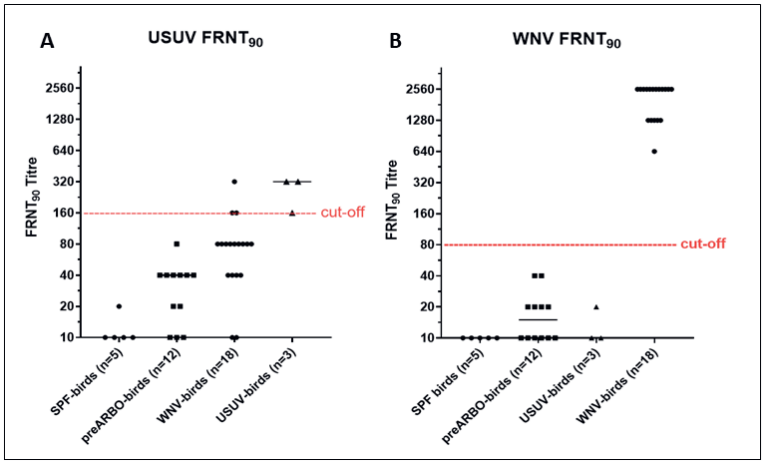
Supplementary material

S1. Sensitivity and specificity of the protein microarray for USUV and WNV NS1 binding IgG antibodies (ROC curves)



ROC curve based on median fluorescence values (RFU) on USUV NS1 and WNV NS1 on the protein microarray with estimated sensitivity and specificity relative to corresponding mFRNT confirmed serum samples. Included sera: SPF-TN and preArBO-TN samples as negatives, experimentally infected European Jackdaws (see supplemental Figure S2 panel C) and chicken sera from this study that are mFRNT-confirmed, as positives. (**A**) USUV PMA cutoff ≥3500 RFU at sensitivity and specificity of 94.1% and 94.1% respectively [AUC = 0.99]. (**B**) WNV PMA cutoff ≥3500 RFU at 75.7% and 82.4% sensitivity and specificity [AUC = 0.91]. preARBO-TN = chickens sampled in 2006 before detection of USUV in the Netherlands; USUV-TP = chicken sera, collected in this study, confirmed via PMA and VNT: WNV-TP = samples from PCR and VNT confirmed WNV-positive European jackdaws.

S2. Established mFRNT$_{90}$ cut-offs for USUV and WNV IgG antibody detection in chickens.



S3. Characteristics of samples used in mFRNT validation.

Sample group	Species	PCR	VNT (cut-off ≥16)		Blocking ELISA (Inhibition %) cut-off ≥40
			WNV	USUV	
SPF-TN	*G. domesticus*	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
preARBO-TN	*G. domesticus*	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
	G. domesticus	NT	NT	NT	NT
WNV-TP	*G. domesticus*	positive	NT	NT	NT
	G. domesticus	positive	NT	NT	NT
	C. monedula	positive	256	<8	NT
	C. monedula	positive	32	<8	85
	C. monedula	positive	256	8	NT
	C. monedula	positive	256	<8	84
	C. monedula	positive	32	<8	84
	C. monedula	positive	16	<8	87
	C. monedula	positive	32	<8	81
	C. monedula	positive	192	<8	89
	C. monedula	positive	32	<8	86
	C. monedula	positive	128	<8	NT
	C. monedula	positive	128	<8	74
	C. monedula	positive	128	<8	75
	C. monedula	positive	32	<8	91
	C. monedula	positive	25	<8	90
	C. monedula	positive	64	<8	89
	C. monedula	positive	384	<8	91
USUV-TP	*G. domesticus*	NT	<8	32	NT
	G. domesticus	NT	<8	20	NT
	G. domesticus	NT	<8	23	NT

(A) USUV FRNT90 cut-off at ≥1:160; SPF-TN = Specific pathogen-free chickens, preARBO-TN = chickens sampled in 2006 before detection of USUV in the Netherlands; USUV-TP = chicken sera, collected in this study, confirmed via PMA and VNT. **(B)** WNV $FRNT_{90}$ cut-off at ≥1:80: WNV-TP = samples from PCR and VNT confirmed WNV-positive European jackdaws (228)* and chickens. **NB**: the red line intercepting the y-axis represents the cut-off limit. (C) Overview of samples used as positives (*TP) and negatives (*TN) to validate of the mFRNT. **NB**: samples tested on blocking ELISA were with the INgezim WNV Compac® commercial assay**.**

Chapter 6.

Rapid response screening for emerging zoonotic pathogens, barriers and opportunities: a study for enhanced preparedness of the Netherlands

Kiki Streng*[1], Pauline A. de Best*[2,3], Aura Timen[3,4,5], Marion P. G. Koopmans[2], Wim H. M. van der Poel[1,6], Reina S. Sikkema[2,7]

* Authors contributed equally

Published in One Health (2023)

Affiliations
[1] Quantitative Veterinary Epidemiology, Wageningen University and Research, Wageningen, the Netherlands
[2] Viroscience, ErasmusMC, Rotterdam, the Netherlands
[3] National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[4] Department of Primary and Community Care, RadboudUMC, Nijmegen, the Netherlands
[5] Athena Institute, VU University, Amsterdam, the Netherlands
[6] Wageningen Bioveterinary Research, Lelystad, the Netherlands
[7] Centre for Avian Migration, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands

Abstract

Background: Outbreaks of zoonotic emerging infectious diseases (EIDs) require rapid identification of potential reservoir hosts and mapping disease spread in these hosts to inform risk assessment and adequate control measures. Animals are often understudied when a novel EID is detected in humans and acquisition of animal samples is hampered by practical, ethical, and legal barriers, of which there is currently no clear overview. Therefore, the three aims of this study are (1) to map potentially available collections of animal samples, (2) to assess possibilities and barriers for reuse of these samples and (3) to assess possibilities and barriers for active animal and environmental sampling in the Netherlands.

Methods: A literature search was performed to identify ongoing sampling activities and opportunities for reuse or active sampling. Semi-structured interviews with stakeholder organizations were conducted to gain further insight into the three research questions.

Results: Various sample collections of surveillance, diagnostic and research activities exist in the Netherlands. Sample size, coverage, storage methods and type of samples collected differs per animal species which influences reuse suitability. Organizations are more likely to share samples, for reuse in outbreak investigations, when they have a pre-existing relationship with the requesting institute. Identified barriers for sharing were, amongst others, unfamiliarity with legislation and unsuitable data management systems. Active sampling of animals or the environment is possible through several routes. Related barriers are acquiring approval from animal- or property owners, conflicts with anonymization, and time needed to acquire ethical approval.

Conclusion: The animal sample collections identified would be very valuable for use in outbreak investigations. Barriers for sharing may be overcome by increasing familiarity with legislation, building (international) sharing networks and agreements before crises occur and developing systems for sample registration and biobanking. Proactive setting up of ethical approvals will allow for rapid animal sample collection to identify EID hosts and potential spillovers.

Definition of terms

Mandate: The delegated power to make decisions in the name of an administrative body. In this study, mandate implies: the decision of the Netherlands Food and Consumer Product Safety Authority (NVWA) to perform sampling on behalf of the Ministry of Agriculture, Nature and Food quality (229).

State supervision: Actions that can be performed by veterinary services (230), in the Netherlands this is the Food and Consumer Product Safety Authority (NVWA), in order to request samples or data from a laboratory or actively take samples at an (suspected) outbreak location (231).

Survey: a component of a surveillance system to systematically collect information with a predefined goal on a sample of a defined population group, within a defined period (232).

Introduction

During the past decades multiple outbreaks of emerging zoonotic diseases have occurred worldwide, including the most recent example of the COVID-19 pandemic (6). Pathogens causing zoonotic diseases pose a threat to human, animal and environmental health. With over 60% of all emerging infectious diseases (EIDs) in humans having a zoonotic origin, early identification of potential (reservoir) hosts, is one of the essential steps to prevent further spread of EIDs (6,233). Furthermore, spill-back of a pathogen from humans to animals may occur, highlighting the need for rapid screening of key (potential) target species. This is important as undetected circulation may give rise to the emergence of new variants and potentially long-term circulation of pathogens within ecosystems (234,235). Rapid detection and origin investigations are pivotal to implement further response and control measures, in order to reduce human and animal morbidity and mortality. Additionally, economic and societal impacts may be averted.

To adequately inform risk-assessment and response measures, One Health outbreak investigations should be designed strategically, depending on the specifics of the outbreak, and be performed timely, risk targeted, with sufficient coverage, sample size and metadata, and appropriate sampling and storage methods (234). Whilst this is widely recognized by the Quadripartite organizations (FAO, WHO, UNEP and WOAH), implementation is still hampered or slowed down (236–243). Additionally, EID outbreak response most often is focussed on humans. For instance, there is sporadic systematic surveillance of SARS-CoV-2 infections in potential animal host species, which can result in late detection of ongoing transmission (244). When SARS-CoV-2 circulation in white-tailed deer was discovered in the USA, the virus had already widely

spread in multiple states (245,246). Mutations were detected in SARS-CoV-2 isolates from white-tailed deer, including a mutation in the receptor-binding motif, and possible spill-back from deer to humans was suggested (247). The potential implications of this spill-back highlight the importance of a One Health focus on rapid EID outbreak response (235). At the moment, animals are often understudied when a novel EID is detected in humans. Reuse of these existing samples not only allows for retrospective surveys to investigate the origin of an outbreak and time of emergence, but also limits the need of active sampling, which can reduce costs as well as animal handling and discomfort (248,249). Testing of existing samples at the same (reference) laboratory also assures comparability and validity of results. Acquiring appropriate animal samples for rapid EID outbreak response can be achieved by active sample collection or possibly by use of existing samples, collected for other purposes. However, the acquisition or collection of animal samples is often hampered by practical, ethical and legal barriers, of which there is no overview available (238–243).

Identifying and overcoming these barriers is of particular relevance to the Netherlands as the country is vulnerable to EID outbreaks due to dense human- and livestock populations, international travel and transport hubs and a water-dominated landscape (250). Previous outbreaks of EIDs in the Netherlands that involved both animals and humans, are for example Q-fever, avian influenza, COVID-19 and West Nile virus. For that reason, we chose the Netherlands as a case study for this investigation. Barriers for rapid response need to be identified before solutions can be suggested. Therefore, the three aims of this study are: (1) To map potentially available collections of animal samples in the Netherlands, (2) to assess possibilities and barriers for reuse of existing collections of animal samples and (3) to assess possibilities and barriers for active animal and environmental sampling in outbreak situations. In this way, this study can provide input for other countries to assess and improve their response to EID outbreaks on the human-animal interface.

Methods

In order to identify barriers which could hamper timely animal testing in case of an EID outbreak, we assessed the literature to identify previously described barriers for active sample collection or sample sharing as well as other barriers for timely research into animal reservoirs. Following the literature search, interviews were held to discuss the extent of identified barriers in practice and to identify potential additional barriers or possibilities for sample reuse or active sample collection. In this way we provide insight into the barriers that need to be addressed concerning EID detection.

Literature search

Literature searches were performed to assess current knowledge on our three research aims and serve as input for interview questions. The searches were carried out via two databases (Embase and Medline). Inclusion and exclusion criteria are listed in table 1 and all search terms can be found in Annex A. Duplicates were removed and titles and abstracts were screened by two authors independently (in EndNote 20.0.1). Full text analysis of literature potentially suitable for inclusion after title and abstract screening was performed by two independent authors, any conflicts were resolved through discussion. Additionally, grey literature was obtained via personal communication and interviews as described later. Legislation applicable to either reuse or active collection of samples was identified via the Dutch national legislative database and included in the analyses. For research question one, full text documents were screened for the identification of sample collections and their characteristics including: surveillance objective(s), pathogen, coverage, sample size, sample type, storage method and storage period and metadata availability.

Table 1. Inclusion and exclusion criteria for literature searches.

(1) Potentially available collections of animal samples in the Netherlands	(2) Possibilities and barriers for reuse of samples	(3) Possibilities and barriers for active sampling
	Inclusion criteria	
Full-text available	Full-text available	Full-text available
Published between January 2011 and July 2021	Published between January 2011 and July 2021	Published between January 2011 and July 2021
English or Dutch	English or Dutch written text	English or Dutch written text
	Articles describing possibilities and/or barriers for sharing animal samples between two (or more) organizations	Studies about physical animal or environmental sample collection in response to an outbreak
		Studies describing legal, practical, ethical, or other barriers/gaps/constraints for physical sample collection
	Exclusion criteria	
Cross-sectional or experimental studies / surveys	Human studies	Studies about human sampling
Studies about human samples or questionnaires	Studies describing sharing of datasets with animal/human health data and/or data related to infectious diseases, rather than physical samples	Studies describing sharing of datasets with animal/human health data and/or data related to infectious diseases, rather than physical samples
Surveillance activities <4 years in duration	Not related to an outbreak	Articles about costs of sampling
Project based sampling activities <4 years in duration	Experimental studies	Articles not describing gaps/barriers/opportunities/constraints relevant to the Netherlands (e.g., wild apes, pastoralist systems)
Studies outside the Netherlands		

Interviews

Semi-structured interviews were conducted by two authors, in two rounds. Round one was focused on research questions one and two. Round two was focused on research question three. Interviews were conducted between July 2021 and February 2022 and lasted approximately one hour. When requested, interview guides were shared with the organization(s) beforehand. Organizations were emailed with follow-up questions or when subjects needed further clarification after the interviews.

Round 1

In order to assess availability of identified sample collections for outbreak investigations, semi-structured interviews were conducted with relevant stakeholders defined as: *organizations that are involved in collection and/or testing of animal samples and can affect and/or are affected by the process of collection and sharing of samples*. Stakeholders were identified based on the Dutch Zoonoses Structure, a Google search for veterinary laboratories and snowballing (251). Fifteen key stakeholder organizations were identified: private laboratories and slaughterhouses (5, of which one through snowballing); organizations performing statutory tasks (5); a university medical centre (1) and poultry, pig and cattle sector representatives (3). Via e-mail, all 15 organizations were invited and asked to appoint one or two representatives for an online interview. The appointed representatives were interviewed using a standardized set of four closed and eleven open (follow-up) questions. Questions were directed to verifying the overviews of ongoing sampling activities and to identify opportunities, conditions and barriers for sharing in case of an EID outbreak. Legal implications for sample sharing, identified in legislation and during interviews, were discussed with three legal experts belonging to three of the invited organizations carrying out statutory tasks.

Round 2

To assess opportunities for timely active collection of animal or environmental samples as part of the response to an outbreak, with emphasis on legislation, semi-structured interviews were conducted with relevant stakeholders defined as: *organizations that are involved in active animal or environmental sample collection and/or can affect or are affected by the process of collection and testing of samples*. Eight relevant stakeholders were identified: Organizations performing statutory tasks (3); the Dutch Zoonoses Signalling Structure (251) (1); the animal ethical board (1) and sector representatives (3). All organizations were invited and asked to appoint one or two representatives for an online interview. Interview questions focused on legislative opportunities, criteria and barriers for active collection of animal and environmental

samples. Questions were derived from findings in legislation and information obtained during the first round of interviews.

Data analysis

To assess barriers and facilitate rapid identification of existing animal sample collections for reuse in EID outbreak, identified sample collections and their characteristics were compiled in a table per animal species. This overview was verified, supplemented and if necessary, altered based on interview results. The overview of sample collections was restricted to longitudinal sampling activities, as it was infeasible to get a complete overview of all short-term (project based) studies because of the numerous organizations involved and since a lot of these studies are not published in (grey) literature.

Interview results were compiled (in Microsoft Excel) and analysed to identify conditions and barriers for reuse of samples and active sample collection. Conditions were defined as: *requirements for sharing or active collection of samples mentioned by at least two stakeholders*. Barriers were defined as: *reasons or circumstances that could hamper reuse or active collection of samples, mentioned by at least two stakeholders*. Literature, legislation and interview results were analysed to identify all options for setting up rapid response surveys, the options were summarized in a decision tree figure, which can be found in Annex C.

Results

Animal sample availability and characteristics

The literature search for potentially available animal sample collections in the Netherlands resulted in 56 papers. Of those, only one fitted the inclusion and exclusion criteria. This paper mainly described wildlife surveillance activities (114). Two documents describing livestock surveillance were found in grey literature (252,253).

An overview of sampling activities, available samples and their characteristics is shown in table 2 (see Annex B for expanded results). This overview includes samples collected for surveillance, diagnostics (including autopsies), and longitudinal surveys involving slaughterhouses or hunters. For wildlife, both opportunistic and targeted sampling activities were identified which were performed by governmental or academic institutions (114). For livestock, sample collection was mostly performed as part of surveillance programs with varying objectives and for a wide range of pathogens (252).

During round one, online interviews were completed with eleven of the 15 invited organizations. One organization did not respond, and all three sector representatives

declined the interview invitation. During round two, online interviews were completed with four of the eight invited organizations. Additionally, one organization responded to our questions via e-mail. Sector representatives decided not to take part in the interview study.

Interviews revealed companion animals (horses and pets) are only sampled for diagnostic purposes. Anecdotal sampling of zoo animals was mentioned during interviews. Sample sizes were lower for wildlife (<1000 per month) than for companion animals (>1000 per month) and livestock (>2000 per month) with highly variable storage times. Wildlife samples are often stored for a long period (≥1 year) whereas diagnostic or surveillance samples of companion animals and livestock generally are kept for short term storage only (≤1 month), except for positive samples. For these species, respondents indicated they are able to retain new samples coming in via the existing sample streams if requested, allowing for reuse of these samples. Availability of metadata such as location, date of sampling, age, sex, and vaccination status varies depending on the species. For wildlife, data on location, sampling date and species are generally available. For diagnostic samples, metadata availability depends on the sample submission forms which are not always filled out completely.

Table 2. Species specific overview of animal samples currently collected in the Netherlands.

***Surveillance:** routinely collection of samples to obtain information about a particular pathogen, or antibodies against this pathogen, in a (targeted) part of the population. **Pathology:** monitoring of the disease status, either for a particular pathogen or unknown causative pathogen, of the populations studied, using samples collected from dead animals. ****Surveillance protocols:** Monitoring disease free status: programs in compliance with Part 2 of Regulation (EU) 2016/429 to confirm disease-free status for one or more listed diseases; Early-warning: systems which identify signals from different sources to indicate the emergence of (new) pathogens; Vaccination status: determine antibody titres after vaccination in accordance with legislation (for Newcastle Disease); Monitoring prevalence: In regular intervals (depending on the pathogen and the operating status of the farm) samples of herds are tested for the occurrence of antibodies or antigen against the targeted pathogens**.** **Spatial coverage:** Clustering, >50% animals within this species category are kept in 3 (or less) provinces of the Netherlands (total 12 provinces); specific regions, animal species are only present in a specific region in the Netherlands (254). **Sample types:** S (serum), B (blood), Sw (rectal/cloacal and/or throat swabs), F (faeces), O (organs/organ tissue) **Anonymization:** sample cannot be traceable to a person (often two number postal code region is used). A more detailed overview is available in Annex B.

	Livestock			**Companion animals**		**Wildlife**		
	 Poultry	 Ruminants	 Pigs	 Horses	 Pets	 Wild birds	 Other	 Wild boar
Sampling characteristics								
Surveillance / other sampling strategy*	Yes + pathology	Yes + pathology	Yes + pathology	No (diagnostic)	No (diagnostic)	Yes + pathology	No (pathology)	Yes

Table 2. Continued

Objectives of surveillance/ monitoring protocol(s)**	1. Monitoring free status 2. Early-warning 3. Vaccination status	1. Monitoring free status 2. Early-warning 3. Monitoring prevalence	1. Monitoring free status 2. Early-warning 3. Monitoring prevalence	NA	NA	1. Early-warning	NA	1. Monitoring free status 2. Early-warning
Examples of pathogens/ diseases in surveillance or monitoring	*Salmonella*, avian influenza, Newcastle disease	Bluetongue, bovine spongiform encephalopathy, *Brucella*	Classical- and African swine fever, *Salmonella*	NA	NA	Avian influenza, arboviruses	NA	Classical- and African swine fever, Aujeszky's
Spatial coverage	All provinces (clustered)	All provinces (clustered)	All provinces (clustered)	All provinces	All provinces	All provinces	Specific regions Depending on species	Specific regions
Sample size	>20.000/month	>5000/month	>2000/month	>1000/month	>2000/month	500-1000/month	<100/month	+-100/month
Sample characteristics								
Sample type(s)	S, B, Sw, F, O	B, S, M, O, F	S, O	B, S, Sw, F, O	B, S, Sw, F, O	S, Sw, O	S, O, Sw, F	B, O, S
Storage method & time	1 week – 1 month (positive samples 1 year - infinity), cooled (frozen -70/-80°C)	1 week – 1 month (positive samples 1 year - infinity), cooled (frozen -70/-80°C)	1 week – 1 month (positive samples 1 year - infinity), cooled (frozen -70/-80°C)	<2 weeks, cooled (4-8°C)	<2 weeks, cooled (4-8°C)	>1 year, frozen (-20/-80°C)	>1 year, frozen (-20/-80°C)	>1 year, frozen (-20/-80°C)
Metadata available	Location, species, sampling date	Location, species, sampling date	Location, sampling date	Variable (not mandatory)	Variable (not mandatory)	Location, species, sampling date, age, sex, disease status	Location, species, sampling date, age, sex, diseases status	Location, sampling date, age, sex
Anonymization required	Yes	Yes	Yes	Yes	Yes	No	No	No

Conditions and barriers for sharing

The second literature search for possibilities and barriers for sample reuse, initially resulted in 48 papers. None of these papers passed the inclusion and exclusion criteria, as they were focused on either biobanking, data sharing or experimental studies and none of them described sharing of physical samples between organizations. Physical sharing of samples is essential if reuse involves for instance testing for newly discovered pathogens, or when specific expertise for testing is required.

If samples are available and suitable for outbreak investigations, organizations must be willing and able to rapidly share. All respondents indicated they would in principle be willing to share samples for EID outbreak response, but noted it is unclear who would be responsible for initiating this process. Options for sharing were mentioned to be case dependent. The conditions and barriers indicated by respondents are listed in table 3. There were some clear differences for conditions and barriers between organization types. Authorship on future publications was not always a condition for private companies in contrast to organizations performing statutory tasks and the university medical centre. Proportionality between relevance of the research question with the sample value was not an issue for private companies as their flow of samples is large and samples would be destroyed anyway. For organizations storing wildlife samples, limited numbers and high value of samples lead to high scrutiny regarding the research relevance. Private companies have less, or sometimes even no experience with Material Transfer Agreements (MTAs) for sharing samples, and thus require more time setting these up.

All respondents indicated that absence of a pre-existing relationship with the organization requesting the samples would restrict sharing options or result in a more time-consuming sharing process. Next to the listed conditions in table 3, permission from third parties was mentioned as a prerequisite for sharing in specific situations. For example, governmental approval may be necessary for sharing samples collected as part of (partly) government funded surveillance or monitoring programs. When samples are tested for notifiable diseases, laboratories indicated coordination with the Netherlands Food and Consumer Product Safety Authority is endorsed since positive results will demand their response. This could also lead to a restriction of the pathogens for which testing is permitted. Resistance to participation among livestock farmers was mentioned, mainly because of fear of negative publicity in case results are (incorrectly) presented in the media. This resistance could lead to restrictions in sharing. Finally, respondents mentioned livestock sectors as important stakeholders to consult when they financially contributed to sample collection. Although sectors do not have the legal ability to decide about sharing samples of farmers, as individual

farmers have to give permission, they represent the sector in discussions with the government and their judgement on cooperation can influence the willingness of individual farmers to cooperate. Sector representatives were identified and invited as stakeholders but as they decided not to take part in our study, we cannot include their views. For wildlife there are less concerns about approval from third parties since no animal owner rights are involved. Furthermore, two private laboratories mentioned the shipment of all samples abroad, with one of them mentioning mandatory destruction of samples within one week. Storage of samples in a High Containment Unit, mentioned by one organization, results in an obstruction for rapid access to these samples.

Table 3. Conditions and barriers for acquiring samples from different organizations.

	Number of organizations for which conditions and barriers apply (total n=11)
Conditions for sharing	
Pre-existing relationship with requesting organization	11 (100%)
Restrictions on pathogens tested for	11 (100%)
Authorship on future (scientific) publication	8 (72.7%)
Proportionality relevance research with sample value	5 (45.5%)
Barriers for sharing	
*Experience with MTAs**	9 (81.8%): Yes, but MTAs have to be adjusted per case. 2 (18.2%): Very limited/no experience with MTAs.
Data management system does not allow for selection of samples (i.e., by region)	4 (36.4%)
Sample retrieval (time) dependent on staff availability	10 (90.9%)

* Material Transfer Agreement

Legal possibilities and barriers

Even though metadata might be available, it cannot always be shared with other organizations. To adhere to the General Data Protection Regulation (GDPR), samples from domestic animals have to be anonymized prior to sharing, unless each individual owner gives permission. As acquiring individual approval is often infeasible, the location is removed from the metadata or transferred into a two-digit postal code (255). Legal experts indicated this anonymization conflicts with the Animal Health Law in case of notifiable diseases, since notification requires the exact location of the sampled animal (256). Six organizations, both private and academic, indicated their unfamiliarity with privacy regulations, especially for notifiable diseases. As a result, samples might be anonymized at a higher level than legally necessary because of precautions taken by the sharing organization. Additionally, pathogens allowed to be tested for might be restricted and setting up MTAs will be more time consuming.

Legislation and interviews with legal experts revealed the Dutch government has the legal possibility of enacting state supervision to enforce organizations to share data or samples (231). This act requires a certain level of urgency and proportionality, which can lead to discussions with involved stakeholders. Because of these discussions, the act is seldom applied. How often this act has been executed during the past decade is unknown.

Active sample collection

No relevant scientific literature was identified with regards to possibilities and barriers for active sampling in the Netherlands, for which the literature search resulted in 100 articles. Most of the articles discussed human samples, diagnostic methods or mathematical modelling and were therefore excluded.

Diagnostic-, non-invasive- and environmental sampling

Active sampling of animals or the environment can be performed in several ways. For research purposes, there are multiple sampling options for which no ethical approval is needed. When there is a diagnostic purpose, defined by European law as: *"procedures and techniques performed by veterinary surgeons…including taking blood samples from an animal, or animals within a herd, to assist in clinical management e.g., disease diagnosis"*, active sampling of animals is possible with owner approval, but without ethical approval (257). Other legal possibilities for sampling without ethical approval are environmental sampling (e.g., air, dust, water), non-invasive sampling of animals (e.g., feathers) or their products (e.g., eggs, milk), and sampling of dead animals (258). Environmental sampling, such as collection of sewage or surface water, is possible but requires approval from the property owner when sampling on private property. For non-invasive sampling of domestic animals (e.g., feathers), permission from the animal owner(s) is required. Acquiring animal products (e.g., bought in stores) and subsequent testing of these products does not require permission of animal owners. Sampling of dead and hunted wild animals is allowed and possible with the cooperation of hunters.

Ethical approval

Ethical approval is needed for field studies for which a scientific research question is the main objective and in case the animal suffers an equal or greater amount of fear relative to injection of a needle (258). The process of writing the proposal and acquiring the approval often takes up to six months. However, the ethical board indicated that there are possibilities to apply for accelerated approval or an umbrella approval. Currently, One Health surveys to map the sources and spread of newly emerging diseases are considered to be research. Umbrella approvals could potentially be used for the rapid setup of One Health surveys if the required sample- size and type, species

and region can be covered. An umbrella approval is a more general approval that describes a certain research question to be answered and declares why and how many animal samples need to be taken[2]. Accelerated approval is only considered if the outbreak causes 'significant acute societal impact', but is rarely used as governmental mandates will likely be initiated in these cases.

Governmental mandates

In addition to active sampling for research purposes, the government has several options embedded in legislation to perform sampling according to the aforementioned methods, without ethical- or animal owner permission. The Dutch Animal Health Law enables the government to organize active animal sampling by governmental mandate (229). This is solely possible for notifiable diseases. European legislation describes a list of notifiable diseases for European member states (259). Nationally, additional veterinary diseases can be made notifiable by the Chief Veterinary Officer, when advised by the Dutch Zoonoses Advisory group, based on a risk-assessment (260). When such a mandate is enacted, the animal species, number of samples and type of samples to be taken at the (suspected) outbreak location, are determined based on available literature. The actual sampling and transport of specimens is performed according to predefined protocols. Next to this, the Public Health Law enables environmental or non-invasive sampling at (suspected) outbreak locations when the public health authorities suspect a human infection of zoonotic origin, originating from this location. Another option is the enactment of state supervision, which allows for active sampling of animals and the environment for outbreak investigation purposes but restricts sampling to (suspected) outbreak locations. This sampling is performed according to predefined protocols. These last two options are (also) possible for non-notifiable diseases.

Discussion

This manuscript presents an overview of opportunities and barriers for rapid screening of animal populations, using the Netherlands as a case study. Even though this study focused on the Netherlands, our results and recommendations can be extrapolated to other countries and may inspire other countries to conduct similar investigations (261,262). Rapid animal sampling or screening in case of detection of novel or zoonotic human pathogens is essential for outbreak investigations and control.

[2] https://orco.baruch.cuny.edu/wp-content/uploads/sites/36/2021/01/Umbrella-Protocol-Submissions.pdf

Reuse of samples

Multiple existing sampling activities as well as long-term stored samples were identified in the Netherlands, showing potential for reuse. Similar sample collections for surveillance and diagnostic testing are in place in other (European) countries, resulting in an extensive number of potentially useful samples (263). In addition to the identified samples, material from for instance natural history collections or biobanks could be used (248).

However, this study identified several barriers for their use in outbreak investigations. Namely, the absence of a (regularly updated) sample collection overview, the variation in sample storage conditions (time, temperature, which samples are stored) and collection of sample metadata. This is in part related to the organization of surveillance activities in the Netherlands, which consist of a mix of public and private initiatives. Awareness of sample availability is therefore reliant on personal connections and networks. Absence of a network between organizations may lead to delays when samples are needed for outbreak investigation, as development of such networks is challenging in times of crisis especially as it is often unclear who is responsible for initiating this. A similar split in public and private surveillance initiatives exists in other countries, leading to similar barriers (264,265).

When suitable samples are identified, sharing opportunities are influenced by legislation, especially regarding anonymization of sample metadata. Respondents reported unfamiliarity with legislation related to sample sharing, including the GDPR. Due to this unfamiliarity, hesitancy towards sharing may arise, potentially even leading to unwillingness to share, or anonymization of metadata at a higher level than legally necessary. Sample usefulness might decrease due to anonymization of privacy sensitive data such as location of sampling, as important data gets lost. When samples are shared for notifiable disease testing, sample locations cannot be anonymized, and animal owner permission is required. Acquiring permission can be time consuming and difficult to achieve, especially in cases where owners do not acknowledge the potential risk of the pathogen of concern or when they fear economic losses due to public opinion and media attention (261,266). Although we are the first to describe these barriers for physical sample sharing, similar reservations in sharing due to legislation and concerns about negative publicity are described for multiple countries, related to data sharing (241,267). It is therefore expected these barriers will also apply for physical sample sharing in other countries. If these barriers cannot be overcome, active sampling in outbreak response might be required.

Active sampling

We identified multiple options for active sampling, namely, diagnostic, environmental, non-invasive (including dead animals) sampling, and additional sampling via ethical approvals or governmental mandates. Since animal sampling is performed mainly to safeguard public health, both the Animal Health Law, the Public Health Law and GDPR apply and influence sampling possibilities. This can result in unclarity, delays and sometimes hesitancy to initiate sampling. In case active sampling is not enforced by the government, ethical approval is often needed. Acquiring this approval can be a time-consuming process, and thus hamper a rapid response. Since ethical approval for projects involving animals is required in all EU member states, and in multiple countries outside of the EU, this barrier is also relevant in an international context. Environmental and non-invasive sampling is possible without ethical approval, but positive results may conflict with the GDPR if samples can be traced back to one likely source. Especially for notifiable diseases, this could lead to conflicts as further investigations or control measures might be required based on the Public- or Animal Health Law. Conflicts between these laws are thus very context and case dependent. Although legislation might differ between countries, similar complexity due to the involvement of multiple laws is observed, including county-specific legislation (268).

Recommendations

First of all, we recommend to registering public, private and research sample collection activities to create insight into available sample streams for reuse in outbreak investigation. The overview created in this manuscript is a first step to generate more insight herein. Central registration of sample collection activities could aid in the identification of useful samples and might improve consistent metadata collection to improve European animal health surveillance efforts (263). In our research short-term projects were not included because we aimed to develop an overview with continuous streams available for future use. However, short-term projects may provide additional options for reuse of samples when properly registered in a database. As an example, a recently performed cross-sectional serosurvey for *Brucella canis* in the Netherlands, could potentially provide 600 additional dog sera (269).

To assure rapid sharing of the identified sample collections, we also recommend setting up sharing networks or appointing one or more coordinating organizations. To facilitate rapid sharing within these networks, agreements based on broad scenarios can clarify expectations and mutual benefits for all stakeholders involved and increase willingness to share (264,265). For EID outbreak response in an international context, identification of international stakeholders and building of collaboration networks is recommended (114,267).

Besides networks, legislation is also of influence on national and international sharing possibilities. As mentioned before, confusion with regards to legislation can lead to hesitancy to share samples. Internationally, legal barriers have been described for data sharing in SARS-CoV-2 response projects, including components such as the Nagoya protocol (240,270). Familiarization with legislation through education or with help from legal experts when setting up (protocols for) sharing or active sampling could improve rapid response outbreak investigation.

Finally, the identified options for active sampling are mainly directed at livestock outbreak locations and their direct surroundings, except for sampling initiated for research purposes. Consequently, wider investigation of the potential role of wildlife is lacking, risking undetected circulation and spillback of new variants. Therefore, we recommend to facilitate more broad ethical approvals, umbrella approvals, with sufficient flexibility to perform response screening for zoonotic EID outbreak investigation. Recognition of the importance of response surveys among all stakeholders could be a start to investigate how ethical approval can be granted for this kind of research.

The findings of this study have a number of important implications for establishing One Health preparedness. An infrastructure containing sample collection overviews and protocols facilitating sample sharing can prevent delays in the identification of suitable samples for EID research and waste of potentially valuable samples. Long-term stored (historical) samples can provide additional information on spatiotemporal disease spread of EIDs exemplified by the identification of MERS antibodies in archived camel samples (271). Furthermore, historical samples can serve as reference in serological assays by providing a pre-emergence background serological profile. Proactive setting up of ethical approvals will allow for rapid animal sample collection in affected areas to rapidly identify EID hosts and potential spillovers. In this study, we underpin the need and show the way forward to achieve a more rapid response to EID outbreaks to safeguard both human and animal health.

Acknowledgements

The authors acknowledge all interviewed or otherwise contacted organizations for their cooperation and useful input to this chapter.

Supplementary material

Annex A – Literature search terms

1. Identify potentially available collections of animal samples in the Netherlands

Database searches combined the following sets of search terms (in title/abstract/keywords):

1)
Livestock
“farm animal”
Animal
“domestic animal”
“pet animal”
Wildlife
“wild animal”
Zoonos*
Zoonotic
“Animals, Domestic"
"Animals, Wild”
2) near
Surveillanc*
Biosurveillanc*
Sampl*
3)
Netherlands
Holland
Dutch

2. Identify possibilities and barriers for sharing of animal samples during/after outbreaks

Database searches combined the following sets of search terms (in title/abstract/keywords):

1)
Livestock
“farm animal”
Animal
“domestic animal”
“pet animal”
Wildlife
“wild animal”
Zoonos*
Zoonotic
“Animals, Domestic"
"Animals, Wild”
2)
Gap
Gaps
Barrier*

Constraint*
Opportunit*
Possibilit*
3)
Reus*
Sharing
Share
4)
Sample*
Specimen

3. Identify possibilities and barriers for active sampling of animals during/after outbreaks
Database searches combined the following sets of search terms (in title/abstract/keywords):
1)
Livestock
“farm animal”
Animal
“domestic animal”
“pet animal”
Wildlife
“wild animal”
Zoonos*
Zoonotic
“Animals, Domestic"
"Animals, Wild”
2)
Outbreak*
3) near
Investigation*
Response
Surveillanc*
Survey
Sampling
4)
Gap
Gaps
Barrier*
Constraint*
Opportunit*
Facilitator*
Possibilit*

Annex B – Sample collection overviews - Domestic (252)

Table 1. Domestic animal surveillance in the Netherlands. AI = Avian Influenza, MP = multiple pathogens, BT = bluetongue, EBL = Enzootic bovine leukosis, CB = Coxiella burnetti, CSF = Classical Swine Fever, ASF = African Swine Fever, TS = Trichinella Spiralis, BSE = Bovine spongiform encephalopathy, BA = Brucella abortus. * see appendix B.1. Active surveillance: Samples routinely collected to obtain information about a specific pathogen, or antibodies against this pathogen, in a (targeted) part of the population. Passive surveillance: Samples collected from dead animals or animals with clinical disease to obtain information about either a specific pathogen or an unknown causative pathogen.

Species	**Poultry**	**Cattle**	**Small ruminants**	**Pigs**	**Horses**	**Companion animals**
Active surveillance	*Early-warning:* AI: 5/barn, min. 30 per farm; every year/4 months (indoor/free-range) NCD: 30 animals, freq. depending on type of poultry *Monitoring prevalence:* Salmonella: every 3 or 15 weeks Monitoring diseases*: depending on pathogen	*Monitoring free status:* BT: 14-20 animals x 20 NL compartments EBL: 8000 farms/year *Monitoring prevalence:* Focus pathogen**: depending Control programmes***: depending on pathogen	*Monitoring free status:* Brucella: 1475 farms, 13 samples/year *Monitoring prevalence:* CB: 1x/month *Early-warning:* Scrapie: 1500 animals/year	*Early-warning:* CSF/ASF: 1x/month *Monitoring prevalence:* Salmonella: 12 animals every 4 months TS: slaughterhouse sampling *Monitoring free status:* Brucella S.: 1x/year boars at semen coll. centres	TS: slaughterhouse sampling	
Type of sample	*Blood, serum, swabs, shoe covers*	*Blood, serum, bulk milk, ear biopsies*	*Serum, bulk milk, brain*	*Serum, semen, diaphragm*	*Diaphragm*	
Other	Zoonotic pathogens (including emerging) in farm animals: every year 200 farms of a different species *(faeces + nose swabs, species dependent)*					
Passive surveillance	*Early-warning:* 'Peil' veterinarians	*Early-warning:* BSE: suspected animals *Monitoring free status:* BA: in case of abortions				
Type of sample	*Carcasses*	*Brain, blood*				
Other	Animals sent in for pathology (not mandatory)					
Other	Samples sent to laboratories for diagnostics (not mandatory)					
Other	Samples collected by slaughterhouses					

Wildlife (114)

Table 2. Wildlife surveillance in the Netherlands. OVP = Oostvaardersplassen nature reserve. Targeted surveillance: surveillance performed to obtain information about a particular pathogen in a particular host animal population, and generally involves collecting samples according to a statistical or probability-based sampling plan. Non-targeted surveillance: pathological examination of animals found moribund or dead.

Species	**Carnivores**	**Rodents**	**Cervids**	**Lagomorphs**	**Birds**	**Wild ungulates**	**Marine animals**	**Bats**
Targeted surveillance	Fox: hunting	(Live) capture	Hunting		(Live) capture	10 per species / year (OVP) Boar: hunting		
Type of sample	*Intestines, muscle, tissues*	*Serum, tissues*	*Blood (serum)*		*Serum, swabs, feathers*	*Blood (serum), tissue*		
Non-targeted surveillance	Pathology of dead (found/hunted) animals; Where applicable slaughterhouse testing							Human contact animals
Type of sample	*This selection may differ per wildlife species or institute: lungs, liver, spleen, kidneys and brain, as well as serum*							*Brain*

** Focus pathogen

Once every two years, the animal health surveillance steering committee 'Begeleidingscommissie monitoring diergezondheid' selects which diseases are under surveillance. Population: Randomly selected farms (farms that join the relevant health programme are excluded). In the cattle population, prevalence surveys on a number of infectious diseases are conducted. Farms are selected by means of random sampling, and examined mostly serologically either in a bulk milk sample (dairy herds) or a fixed number of animal sera. The choice of pathogens is made by the stakeholders and is mainly based on the zoonotic aspects or economical aspects of infectious pathogens. This surveillance component is pro-active and serves to describe prevalence and trends over time of infectious diseases.

****Cattle control programmes*

Control programmes for Salmonella spp. (bacteria), Leptospira Hardjo, (bacterium), Mycobacterium avium subsp. paratuberculosis, bovine viral diarrhoea virus (BVDV), bovine herpes virus type 1 (BHV 1). In regular intervals (depending on the pathogen and the operating status of the farm), bulk milk samples, blood samples or ear biopsy samples of dairy herds are tested for the occurrence of antibodies or antigen against the targeted pathogens. Depending on the results and the pathogen, dairy herds are categorized as free of disease, suspected of disease or infected.

Appendix B.1 (114,252)

*Poultry monitoring diseases:

Pathogen	Type of poultry	When/how often are samples taken	Sample type(s)	Amount of samples
S. Pullorum and S. Gallinarum	(all) reproduction stock	20-22 weeks old	blood samples	from 1%, min 30; max 60
S. arizonae (same system as zoonotic salmonella)	1.Egg producing breeders 2.Rearing poultry 3.Layers 4.Broilers 5.Hatchery	1.Every 2-3 weeks 2. <3 days old, 28 days, 2 weeks before movement to production farm 3. Every 15 weeks & max. 3 weeks before slaughter 4.Before placement & max. 3 weeks before slaughter 5. Every hatch	boot swabs (poultry on litter), manure samples (colony housing), overshoes	manure: min. 300 gram overshoes: 2-5 pairs
Mycoplasma gallisepticum, and M. meleagridis	Grandparent & parent stock	end of rearing period two weeks before placement to production site grandparent - every 8 weeks in production period parent - every 12 weeks in production period	blood samples	from 1%, min 30; max 60
Mycoplasma synoviae	1.Grandparent & layer parent stock not vaccinated 2. Layer parent stock vaccinated 3. Meat parent stock 4.Meat grandparent stock 5. Layers (vaccinated) 6.Meat turkeys (vaccinated)	1. End of rearing period, two weeks before placement to production site 2.& 3. One week before placement on production farm 4. Every 8 weeks in production period 5a. Rearing period 5b. End of production period 6. End of the fattening period	1, 5a,5b&6. Blood samples (tracheal swabs) 2. & 3. tracheal swabs	1, 2 & 5. from 1%, min 30; max 60 2, 5a & 6. 24 per house 5b. 10 (12) per house 3. All sampled

Annex C – Decision tree

This decision tree shows the two identified routes for sample collection: reuse of existing samples or active collection of samples. For each step, criteria and possibilities are shown. This decision tree can be used to identify options to set up rapid response surveys in case of an EID outbreak.

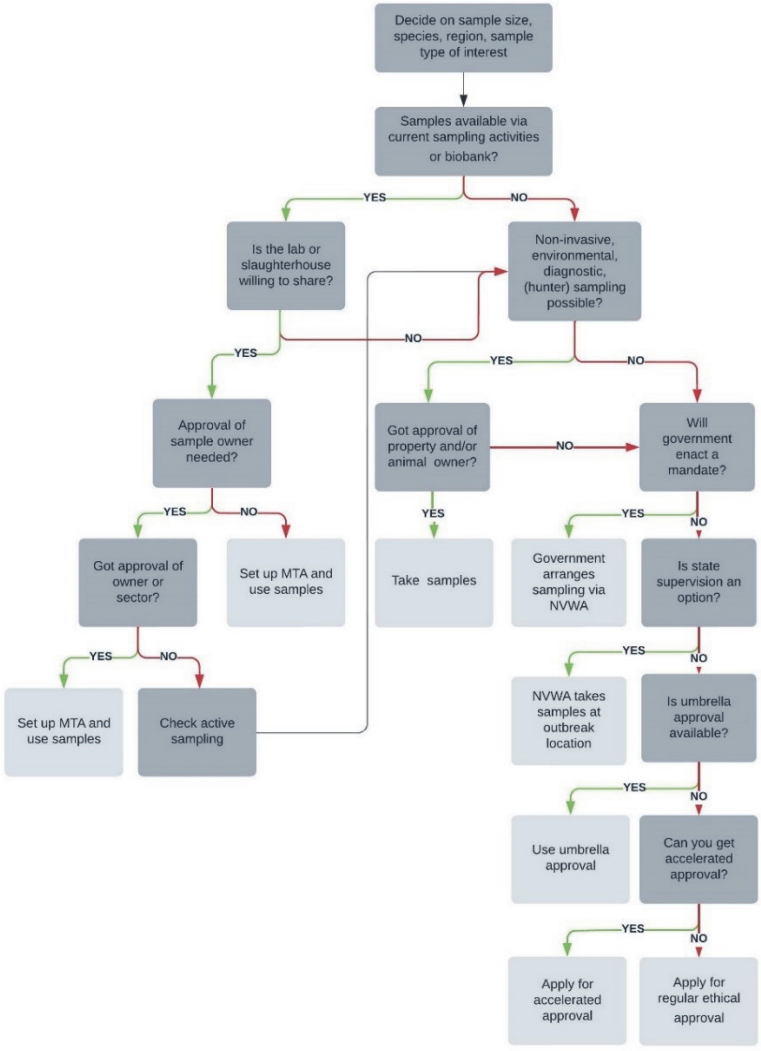


Figure 1. Decision tree for rapid response sampling in animals in the Netherlands (Created with Lucid App). Lab: laboratory; MTA: Material transfer agreement; NVWA: Netherlands Food and Consumer Product Safety Authority

Chapter 7.

The potential of Rift Valley Fever virus (RVFV) outbreaks in current and future scenarios in the Netherlands

Kiki Streng[1], Mariken de Wit[1], Martha Dellar[2], Wim H.M. van der Poel[1,3], Boris V. Schmid[1]*, Quirine ten Bosch[1]*

In preparation

Affiliations
[1] Wageningen University and Research, Quantitative Veterinary Epidemiology, Wageningen, the Netherlands
[2] Deltares, Utrecht
[3] Wageningen Bioveterinary Research, Lelystad, the Netherlands

Abstract

Rift Valley Fever virus (RVFV), is a zoonotic mosquito-borne virus, belonging to the family *Phenuiviridae*, genus *Phlebovirus*. Outbreaks of Rift Valley fever (RVF) pose a threat for human and animal health, as well as the economy. Currently, the virus is present in Africa and the Arabian peninsula. However, inevitable changes in agricultural policies, land use and climate will likely increase the risk of outbreaks in areas like the European Union (EU). The Netherlands is a country in the EU with very high human and livestock densities and was identified as being at highest risk of RVFV introduction in the EU.

This study compares the potential of RVFV outbreaks between a reference and two future scenarios for 2050 in the Netherlands. These scenarios are based on two distinct Shared Socioeconomic Pathways (SSPs). We developed an SEIR-SEI model that includes two vector (*Culex pipiens/torrentium* and *Aedes vexans*) and host (cattle and sheep) species. The ordinary differential equations were used to construct a Next Generation Matrix to derive the basic reproduction number (R_0). R_0 values were calculated across space and time and used to compare the potential for RVFV outbreaks between scenarios.

Our results indicate that the transmission season will likely be prolonged in both future scenarios, especially in SSP5, the "fossil-fuelled" scenario. The daily mean R_0 in the calculated grid cells reaches higher values in SSP5 compared to the other scenarios. Low estimated R_0 values are mainly observed in regions with very low livestock densities, which are especially prominent in May and September. Higher R_0 values are first observed around river systems and wetlands in June, when *Ae. vexans* populations are high. Two possible RVFV introduction locations, Schiphol airport and the Rotterdam harbour, are located in areas with relatively low R_0 values as well as a relatively small number of consecutive days where R_0 is above 1.

Future research is needed to elucidate the size and impact of potential outbreaks of RVFV in the Netherlands. While absolute estimates of R_0 for emerging pathogens should be considered with caution and are highly sensitive to model assumptions, the future trends illustrated in this study are robust to a range of assumptions. Further sensitivity analyses will be needed to assess the robustness of the outcomes with regards to uncertainty and variation in future scenarios and underlying biological assumptions of the model. The results of this study highlight the need for increased preparedness for RVFV due to an increased potential for outbreaks in the future, which includes the development of safe and effective vaccines.

1. Introduction

Plebovirus riftense, previously called Rift Valley Fever virus (RVFV), is a zoonotic mosquito-borne virus, belonging to the family *Phenuiviridae*, genus *Phlebovirus* (12). Rift Valley Fever (RVF), the disease caused by RVFV, affects humans and ruminants such as cattle (*Bos taurus*), goats (*Capra hircus*) and sheep (*Ovis aries*). The causative agent of RVF was first identified in 1930 in a man from Kenya (272). Currently, the virus is present throughout Africa and more recently introduced on the Arabian Peninsula (17). Outbreaks of RVF pose a threat for human and animal health, as well as the economy. There is no human vaccine available, but the virus was prioritized in 2018 by the World Health Organization (WHO) on the list of diseases for which urgent acceleration of R&D is needed (273). Vaccine development is ongoing as RVF is a target disease for the Coalition of Epidemic Preparedness Innovations (CEPI). RVF is listed by the World Organisation for Animal Health (WOAH) and has an A listed status in European Animal Health Law, which means the immediate eradication measures must be taken as soon as it is detected (274).

Transmission of the virus to animals takes place mainly via mosquito vectors, predominantly *Aedes* and *Culex* spp. (275). *Aedes* spp. are known to vertically transmit the virus to their offspring, via eggs that may remain viable for very long periods of drought (173). Flooding of dry areas may lead to explosive increase in numbers of mosquitoes. Therefore, outbreaks are often associated with extreme or increased rainfall. Direct transmission between animals is subject to debate in current literature (275). Young animals are extremely susceptible and mortalities of 70-100% may occur in lambs and kids, with calves being less susceptible (20-70% mortality) (276). In adult animals, the signs of disease tend to be non-specific but abortion rates may approach 85% or 100% in cattle and sheep respectively (276). There is no specific treatment available. Vaccination is the only suggested method to effectively prevent disease in animals. An attenuated vaccine is available for livestock, but may cause foetal abnormalities and abortion in pregnant animals (277). New vaccines are being developed, but no vaccine is currently registered for use in the EU (275,278).

While serological studies have not detected RVFV infections in ruminants in the European Union (EU) (279,280), a number of studies report the potential threat of introduction and spread of RVF to the EU. Changes in climate, and travel as well as trade of animals or animal products have been mentioned as potential drivers for introduction of RVFV (16,281–283). Risk evaluation by Dufour et al. (284) estimated a moderate to high impact of RVF on animal health and the economy. Risk factors for local transmission and establishment described in literature are often related to the abundance of competent vectors, hosts, and climate conditions (285). The risk of local transmission within the EU was assessed as moderate by the European Food Safety

Authority (EFSA) in 2020. This classification is based on the presence of competent vectors and full susceptibility of all hosts within the EU. The probability of establishment varies per country but ranges from moderate to very high (283).

The Netherlands is a country in the EU with very high human and livestock densities. The country has a lot of water rich areas (deltas, wetlands) and competent RVFV vectors are widely present (35,200). The RVFV introduction risk by animal trade is assumed very low (283). The probability of RVFV entry via infected mosquitoes is considered medium, as the Netherlands has large transport and travel hubs like Schiphol airport. Therefore, the Netherlands was identified as being at highest risk of RVFV introduction in the EU (283). Reduction of livestock numbers has been topic of debate to reduce the impact on climate and emissions. Even though discussion on the differences between intensive livestock production versus extensive or organic farming with regards to spread of mosquito-borne viruses is ongoing, no concrete knowledge based on modelling or literature exists.

Currently, no studies have investigated the difference between future scenarios (climate, land use, agricultural policies) on the potential of RVFV outbreaks. This is important, especially for countries like the Netherlands facing the highest introduction risk of the EU, combined with the high human and livestock densities and no registered human nor animal vaccines. Inevitable changes in agricultural policies, land use and climate will likely affect the risk of major outbreaks in the future. In order to be prepared for future scenarios, we aim to compare the potential of a RVFV outbreak between a reference and two future scenarios for 2050 in the Netherlands. We construct a Next Generation Matrix (NGM) to derive the basic reproduction number (R_0) across space and time. This is used to compare the potential of RVFV outbreaks between scenarios. R_0 estimates provide information about the potential vulnerability to and scale of future outbreaks. We calculated R_0 for each day and location to explore variations in outbreak risk across the year and between regions. High R_0 values indicate a higher risk of major outbreaks, which would require more rigorous control measures.

2. Methods

We developed an SEIR-SEI model that includes two vector and two host species (Figure 1) and derived the NGM in order to obtain the daily R_0 estimates for each 1 km^2 grid cell. To study the potential effects of changes in climate, land use, and livestock farming, outcomes of two future scenarios were compared with a reference scenario. The two future scenarios represent hypothetical situations of the Netherlands around 2050. An overview of the input data, data handling, model structure and outcome measures is shown in Figure 1.

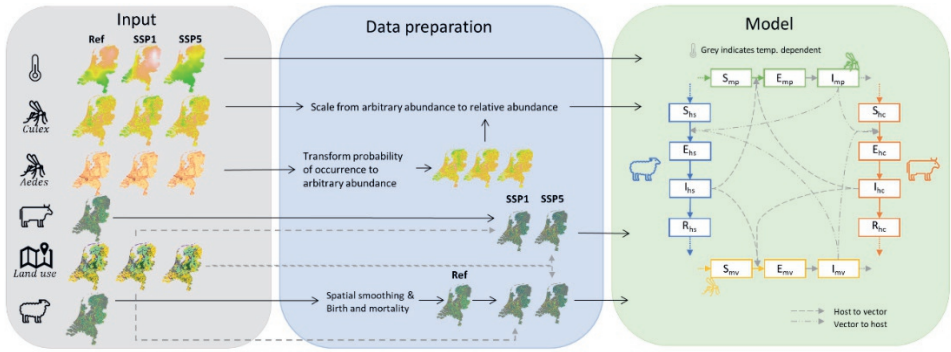


Figure 1. Overview of the input data (daily mean temperature, daily *Culex pipiens/torrentium* arbitrary abundance, daily probability of occurrence *Aedes vexans*, cattle abundance, land use and sheep abundance), data preparation and the SEIR-SEI model.

2.1 Model structure and assumptions

Culex pipiens/torrentium and *Aedes vexans* (Meigen) were included as competent RVFV vectors in the model, as both are present in the Netherlands (24,286). Vectors are categorized into three states: susceptible (S_{mp} for *Cx. pipiens/torrentium* and S_{mv} for *Ae. vexans*), exposed (E_{mp} *and* E_{mv}) or infectious (I_{mp} *and* I_{mv}). Two competent host species, cattle (*Bos taurus*) and sheep (*Ovis aries*), are categorized as susceptible (S_{hc} for cattle and S_{hs} for sheep), exposed (E_{hc} and E_{hs}), infectious (I_{hc} and I_{hs}) and recovered (R_{hc} and R_{hs}) (Figure 1). The total number of cattle, sheep, *Cx. pipiens/torrentium* and *Ae. vexans* are indicated by N_{hc}, N_{hs}, N_{mp} and N_{mv}, respectively. Direct transmission between competent hosts was not included in the model. Vectors and hosts were assumed to mix homogeneously within each grid cell. Biting rate, extrinsic incubation period and mortality rate of vectors were assumed to be temperature dependent. The proportion of bites on competent hosts (cattle and sheep) for each vector was a function of the total number of competent hosts in each grid cell. Due to their respective biting preferences, *Ae. vexans* had a higher proportion of bites on competent hosts compared to *Cx. pipiens/torrentium* (287,288) (supplementary material S.7). Transmission probability upon infectious bite, intrinsic incubation rate and disease induced mortality rate were kept constant across current and future scenarios.

Daily mean temperature data for the reference scenario was obtained via the Dutch Meteorological Institute (KNMI), for a period of 30 years (1991-2020) (289). Temperature data for future scenarios were based on climate predictions, where daily mean temperatures over the period which were averaged for each day number, based on a 30-year period (2036-2065) (290). Parameter values and functions for temperature dependent parameters, including references, are shown in Table 1. Abundance (on an

arbitrary scale) of *Cx. pipiens/torrentium* was obtained from Krol et al. (291). The probability of occurrence of *Ae. vexans* for all scenarios was obtained from Dellar et al. (292) and transformed to abundance using a generalised additive model as described in the Supplementary Material (S4.1). Livestock census data was obtained from the Netherlands Enterprise Agency (RVO) census from the 1st of April 2022 for both cattle and sheep. Farm location data was transformed into 1km^2 grid structure by calculating the sum of animals located within each grid cell based on coordinates of the farm. Cattle abundance was assumed to remain constant, while sheep abundance increased over the lambing season (Jan-Apr) and declined during slaughter season (Sept-Oct) to return to the same abundance as the 1st of January. Parameter values related to both host types are shown in Table 2. More information on the input data and data preparation steps can be found in the supplementary material S.1-S.5.

Table 1. Parameter values (and functions) vectors. T = temperature (degrees Celsius).

Parameter	Definition	Value / formula	References
α_i	Biting rate of vector *i*	*Cx. Pipiens/torrentium*: $\frac{0.344}{(1+1.231^{(-0.184*(T-20))})}$ *Ae. vexans*: $\frac{1+0.12}{\left(\frac{1}{0.0173*(T-9.6)}\right)}$	(293–295)
ν_i	Proportion of bites of vector *i* on competent hosts	See supplementary material S.7	(287,288)
$\emptyset_{ij}$	Relative preference of vector *i* for host *j*	*Cx. pipiens/torrentium*-cattle: 0.5 *Cx. pipiens/torrentium*-sheep: 0.5 *Ae. vexans*-cattle: 0.8 *Ae. vexans*-sheep: 0.2	*Cx. pipiens*: NA *Ae. vexans:* (296)
θ_{ij}	Proportion of blood meals taken on host population *j*	$\frac{\emptyset_{ij}*N_j}{\emptyset_{is}*N_{hs}+\emptyset_{ic}*N_{hc}}$	(295)
ρ_{ij}	Transmission probability of vector *i* to host *j*	*Cx. pipiens/torrentium* to cattle: 0.94 *Cx. pipiens/torrentium* to sheep: 0.94 *Ae. vexans* to cattle: 0.38 *Ae. vexans* to sheep: 0.38	(286)
ε_i	Incubation rate of vector *i*	*Cx. pipiens/torrentium* (T>13, else 0): $(7.38*10^{-5})*T*(T-11.4)*(45.2-T)^{0.5}$ *Ae. vexans*: $(8.84*10^{-5})*T*(T-9)*(45.9-T)^{0.5}$	(297)
μ_i	Mortality rate of vector *i*	*Cx. pipiens/torrentium* (T<32, else 0.17): $\frac{1}{(-4.86*T+169.8)}$ (+ 0.0714 diapause rate* from Sept 15th) *Ae. vexans*: $\frac{1}{(25.8-0.45*T)}$	(297,298) *supplementary material S.7

Table 2. Parameter values (and functions) hosts and related references.

Parameter	Definition	Value / formula	References
ρ_{ji}	Transmission probability from host *j* to vector *i*	Cattle to *Cx. pipiens/torrentium*: 0.14 Cattle to *Ae. vexans*: 0.14 Sheep to *Cx. pipiens/torrentium*: 0.14 Sheep to *Ae. vexans*: 0.14	(286)
ε_j	Incubation rate host *j*	Cattle: 0.5 Sheep: 0.5	(276)
γ_j	Recovery rate host *j*	Cattle: 0.2 Sheep: 0.2	(298)
μ_j	Mortality rate host *j*	Cattle: 4.48e-4 Sheep: 5.278e-4 (+ 0.0195 for slaughter 7th Sept-27th Oct)	(299) & expert opinion
δ_j	Disease related mortality rate host *j*	Cattle: 0.0176 Sheep: 0.0312	(295,300)

Derivation of the basic reproduction number (R_0)

A daily R_0 was estimated for each grid cell in which (one or both) competent hosts as well as (one or both) vectors were present. First the transmission matrix (T) and transition matrix (Σ) were created based on equations 1-8 of the infected subsystem. The transition and transmission matrix are available in supplementary material S.8 and S.9. These matrices were used to obtain the next generation matrix (NGM), which was constructed according to the method described by Diekmann et al. (301), using $K = -T\Sigma^{-1}$. From the NGM, we derived the daily R_0 for each 1 km^2 grid cell over a one-year period (1st of April until 31st of October).

Culex pipiens/torrentium infected subsystem:

$$\boldsymbol{E_{mp}} = \alpha_p * \nu_p * \theta_{pc} * \rho_{cp} * \frac{I_{hc}}{N_{hc}} * S_{mp} + \alpha_p * \nu_p * \theta_{ps} * \rho_{sp} * \frac{I_{hs}}{N_{hs}} * S_{mp} - \mu_{mp} * E_{mp} - \varepsilon_{mp} * E_{mp} \quad (1)$$

$$\boldsymbol{I_{mp}} = \varepsilon_{mp} * E_{mp} - \mu_{mp} * I_{mp} \quad (2)$$

Aedes vexans infected subsystem:

$$\boldsymbol{E_{mv}} = \alpha_v * \nu_v * \theta_{vc} * \rho_{cv} * \frac{I_{hc}}{N_{hc}} * S_{mv} + \alpha_v * \nu_v * \theta_{vs} * \rho_{sv} * \frac{I_{hs}}{N_{hs}} * S_{mv} - \mu_{mv} * E_{mv} - \varepsilon_{mv} * E_{mv} \quad (3)$$

$$\boldsymbol{I_{mv}} = \varepsilon_{mv} * E_{mv} - \mu_{mv} * I_{mv} \quad (4)$$

Cattle infected subsystem:

$$\boldsymbol{E_{hc}} = \alpha_p * \nu_p * \theta_{pc} * \rho_{pc} * \frac{I_{mp}}{N_{hc}} * S_{hc} - \alpha_v * \nu_v * \theta_{vc} * \rho_{vc} * \frac{I_{mv}}{N_{hc}} * S_{hc} - \mu_{hc} * E_{hc} - \varepsilon_{hc} * E_{hc} \quad (5)$$

$$\boldsymbol{I_{hc}} = \varepsilon_{hc} * E_{hc} - \mu_{hc} * I_{hc} - \delta_c * I_{hc} - \gamma_c * I_{hc} \quad (6)$$

Sheep infected subsystem:

$$\boldsymbol{E_{hs}} = \alpha_p * \nu_p * \theta_{ps} * \rho_{ps} * \frac{I_{mp}}{N_{hs}} * S_{hs} + \alpha_v * \nu_v * \theta_{vs} * \rho_{vs} * \frac{I_{mv}}{N_{hs}} * S_{hs} - \mu_{hs} * E_{hs} - \varepsilon_{hs} * E_{hs} \quad (7)$$

$$\boldsymbol{I_{hs}} = \varepsilon_{hs} * E_{hs} - \mu_{hs} * I_{hs} - \delta_s * I_{hs} - \gamma_s * I_{hs} \quad (8)$$

2.2 Future scenarios

In this study we compare the potential impact of RVFV outbreaks between a 'reference' and two future scenarios. Future scenarios are based on the Dutch One Health Shared Socio-economic Pathway (SSP) scenarios SSP1 and SSP5 as described by Dellar et al. (112). These scenarios are based on global and European SSPs, combined with new insights through (grey) literature research and stakeholder consultation. SSP1 is paired with the RCP2.6 and KNMI low emissions climate scenarios, while SSP5 is paired with RCP8.5 and KNMI high emissions climate scenarios (302). SSP1 and SSP5 were chosen for this study specifically as they represent two distinct agricultural scenarios in which the farming practices, next to environmental factors, are very distinct. Land use maps for these scenarios are based on Dellar et al. (303) and are described in supplementary material S2.

Description of scenarios based on Dellar et al. (112)

SSP1 *"Together green"* is the circular and nature-based scenario. Biodiversity is increased and agricultural land is more closely connected to nature. Organic agriculture is increased while technological innovations allow for higher productivity. Livestock numbers are strongly reduced (-50% cattle) as agriculture becomes more sustainable and less intensive. More attention is given to animal welfare and animal health in general. Extreme weather events are more common than in the reference scenario, but due to the high level of climate change mitigation these events are not as extreme as in SSP5. Deliberate flooding might be executed as a strategy to 'live with water'. There is more space for nature (+3% surface area compared to reference) and urban areas (+2% compared to reference).

SSP5 *"After us comes the deluge"* represents a fossil-fuelled economy scenario. Very limited to no attention is given to sustainability and circularity. Biodiversity decreases and extreme weather events and flooding occur more frequently compared to SSP1. Nature surface area decreases (-2%) compared to the reference scenario, while urban surface area increases (+6%). As the human population increases, there is a big drop in agricultural land and all livestock is concentrated in specific regions. Management practices represent factory-like conditions and no attention is given to animal welfare. This intensification results in increased productivity. Due to this intensification and synthetic proteins becoming more popular, the number of livestock remains fairly constant compared to the current situation (about -10%).

Implementation of scenarios

In SSP1, cattle numbers decrease with 50% (112). Our implementation of this reduction was achieved in two steps. Firstly, we used the reference land use map and the 2022 livestock census data to calculate the distribution of cattle across land use types (urban, pasture, crops, forest and non-forest nature (NFN)). Then, we projected the SSP1 land use map over the 2022 livestock census data. As SSP1 has a large increase in NFN grid cells, many current-day cattle farms "move into" future NFN. As we assume a similar distribution of the cattle population across land use types in SSP1, this resulted in a net reduction of cattle of 70.9% for cattle present in SSP1 urban areas, 38.7% for cattle in pasture, 48.8% for cattle in cropland, and 76.9% and 96.7% for cattle present in forest and NFN, respectively (supplementary material Table S3). Sheep are not as intensively farmed as cattle so massive reductions are not expected for SSP1. The reduction of 6% compared to the reference (supplementary material Table S2), will take place in NFN grid cells, as this land use type increases with 105% in SSP1 and does not accommodate that many sheep (expert opinion). However, sheep will still be used for landscape management in green urban areas, pasture, agroforestry, dunes and on dikes.

In SSP5, cattle numbers remain fairly constant compared to reference (-10%) (112). However, due to the increased human population and recreational areas, many geographical areas are not suitable to agricultural farming in 2050. All cattle are removed from urban, forest and NFN grid cells. An increase (5.4%) of cattle in pasture grid cells is observed as farming becomes more intensive. Small sheep farms (10-20 sheep) are deemed unsustainable due to high land prices and are thus removed. Furthermore, urban areas become more intensively developed resulting in less (green) space for animals. Therefore, an additional 60% reduction of sheep in urban areas was accounted for. This leads to a 13% reduction of sheep compared to the reference. More information on the number of cattle and sheep per land use type can be found in the Supplementary material (Tables S3 and S4).

3. Results

The Netherlands encompasses 35,502km^2 of grid cells that have a land use type (urban, pasture, crops, nature, or non-forest nature). The basic reproduction number (R_0) was estimated for all grid cells with one or both competent hosts and one or both competent vectors. For the reference and SSP1 scenario this resulted in estimates for approximately 77% of the total land surface of the Netherlands, corresponding to 27,261 grid cells. For the SSP5 scenario, results were calculated for a reduced number of grid cells (26,489) due to the lower number of grid cells with competent hosts. Consequently, the SSP5 scenario included 772 fewer grid cells compared to the reference and SSP1 scenario.

Both future scenarios are characterized by an earlier peak in *Ae. vexans* relative abundance and an earlier decline compared to the reference. In SSP5, *Ae. vexans* populations peaked earliest due to the warmer conditions, although the expected range of rising temperatures were also expected to lead to shorter mosquito lifespans, especially in July and August (297) (supplementary material, Figure S10). In contrast to SSP5, peak relative abundances of *Ae. vexans* occurred slightly later in SSP1 (supplementary material, Figure S7b). *Culex pipiens/torrentium* populations peaked at comparable levels in reference and SSP scenarios, yet both SSP5 and SSP1 scenarios exhibited an earlier increase in abundance compared to the reference (supplementary material, Figure S5). The number of sheep fluctuate over the year, but are modestly reduced from ±1.15 million in the reference scenario (on the 1st of January) to ±1.07 million in SSP1 and ±1 million in SSP5. The number of cattle will be more drastically reduced, from ±2.8 million in reference to ±1.4 million in SSP1 and to ±2.5 million in SSP5.

The transmission season is prolonged in both future scenarios (longest in SSP5) (Figure 2). The daily mean R_0 in the calculated grid cells reaches higher values in SSP5 compared to the other scenarios (Figure 2). The proportion of grid cells with an R_0 value above one increases during May, after which it remains constant at about 70% throughout the transmission season (Figure 3). At the start of the transmission season, the R_0 is mainly dominated by *Ae. vexans* for which abundance peaks in June-July. *Cx. pipiens/torrentium* peaks in August and dominates the later part of the transmission season. From the second half of August, a declining trend in mean R_0 starts, and after the 15th of September a sharp decline is observed in all scenarios as we assume this is when diapause of *Cx. pipiens/torrentium* starts , which leads to a rapid decline in host seeking mosquitoes. Across all grid cells, the highest number of consecutive days where R_0>1 was 175 days in the reference scenario, compared to 190 days in the SSP1 scenario and 193 days in the SSP5 scenario. This again indicates a higher potential for (large) outbreaks in the future scenarios, especially SSP5, compared to the reference.

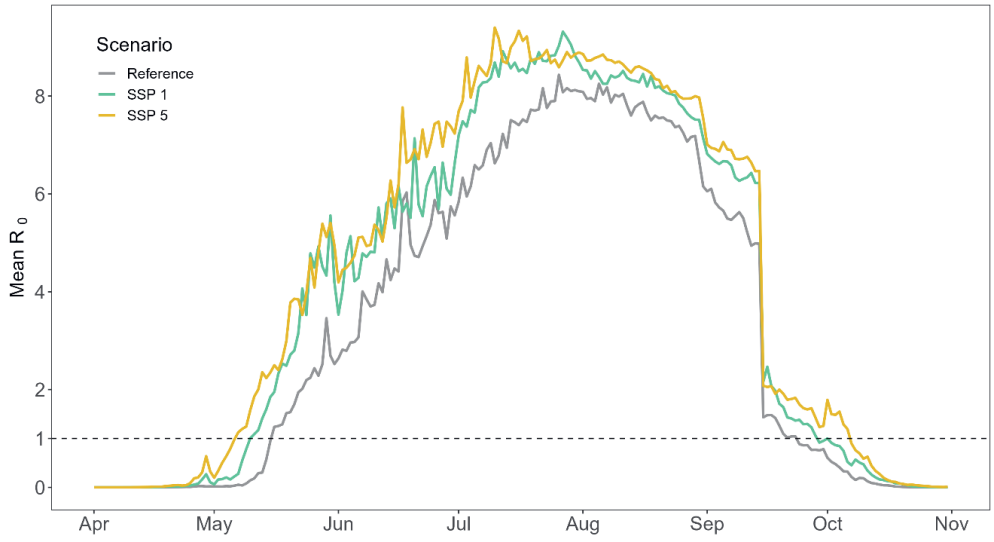


Figure 2. Mean daily R_0 values over all grid cells with competent host(s) and vector(s) (reference and SSP1 have 27,261 grid cells, SSP5 has 26,489 grid cells). Dashed line indicates R_0=1.

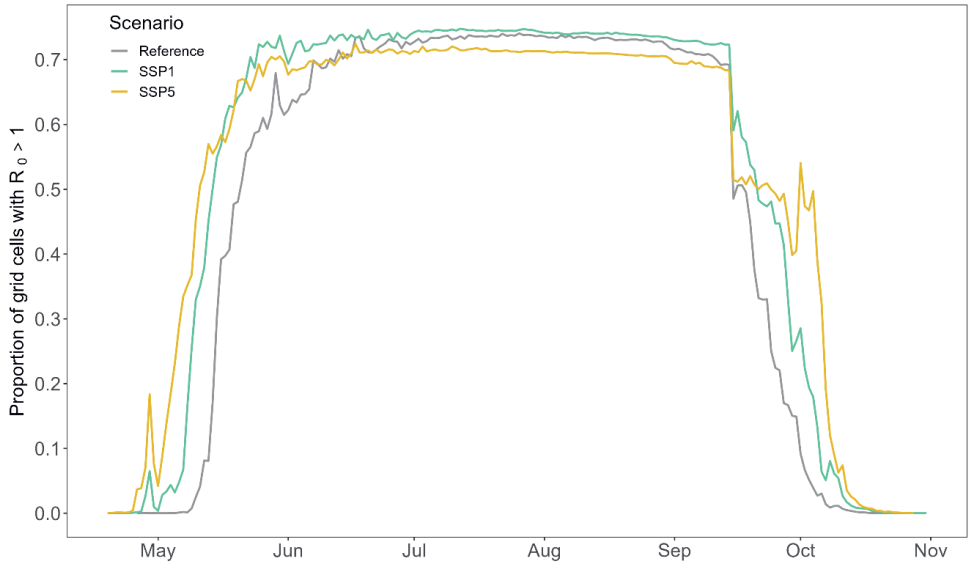


Figure 3. Proportion of land surface area of the Netherlands with an R_0 above 1 over time.

Spatially, we observed relatively similar patterns of R_0 across scenarios over the transmission season (Figure 4). Low estimated R_0 values are mainly observed in regions with very low livestock densities, which are especially prominent in May and September. This finding follows from the assumption that, if few livestock hosts are available, mosquitoes will revert to non-competent hosts for their blood meals. Higher

R_0 values are first observed around river systems and wetlands in June, when *Ae. vexans* populations are high. In July and August, higher R_0 values are predominantly seen in the north and east, where livestock densities are high (Figure 4). Two possible RVFV introduction locations, Schiphol airport and the Rotterdam harbour, are located in areas with relatively low R_0 values as well as the number of consecutive days R_0 is above 1 (Figure 5).

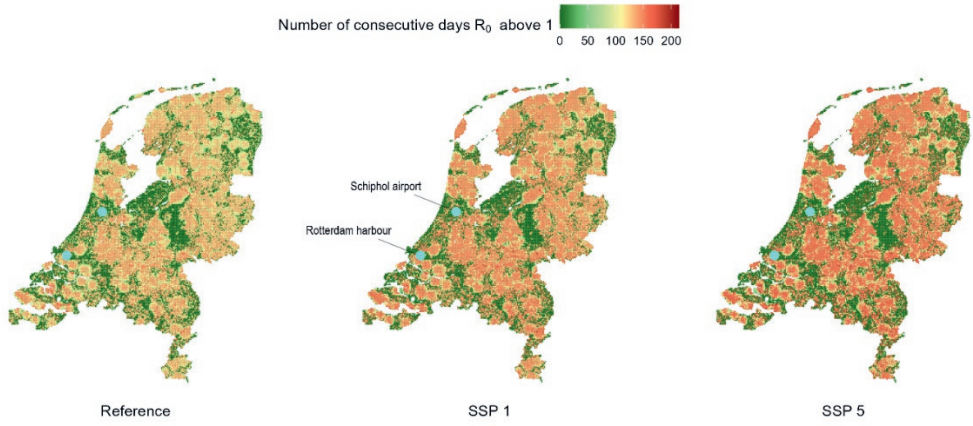


Figure 5. Maximum number of consecutive days of R_0 above 1 within study period (1st of April – 31st of October). Large travel and transport hubs Schiphol airport and Rotterdam harbour are indicated by cyan dots. Cells without competent hosts are shown as green (zero days with R_0>1).

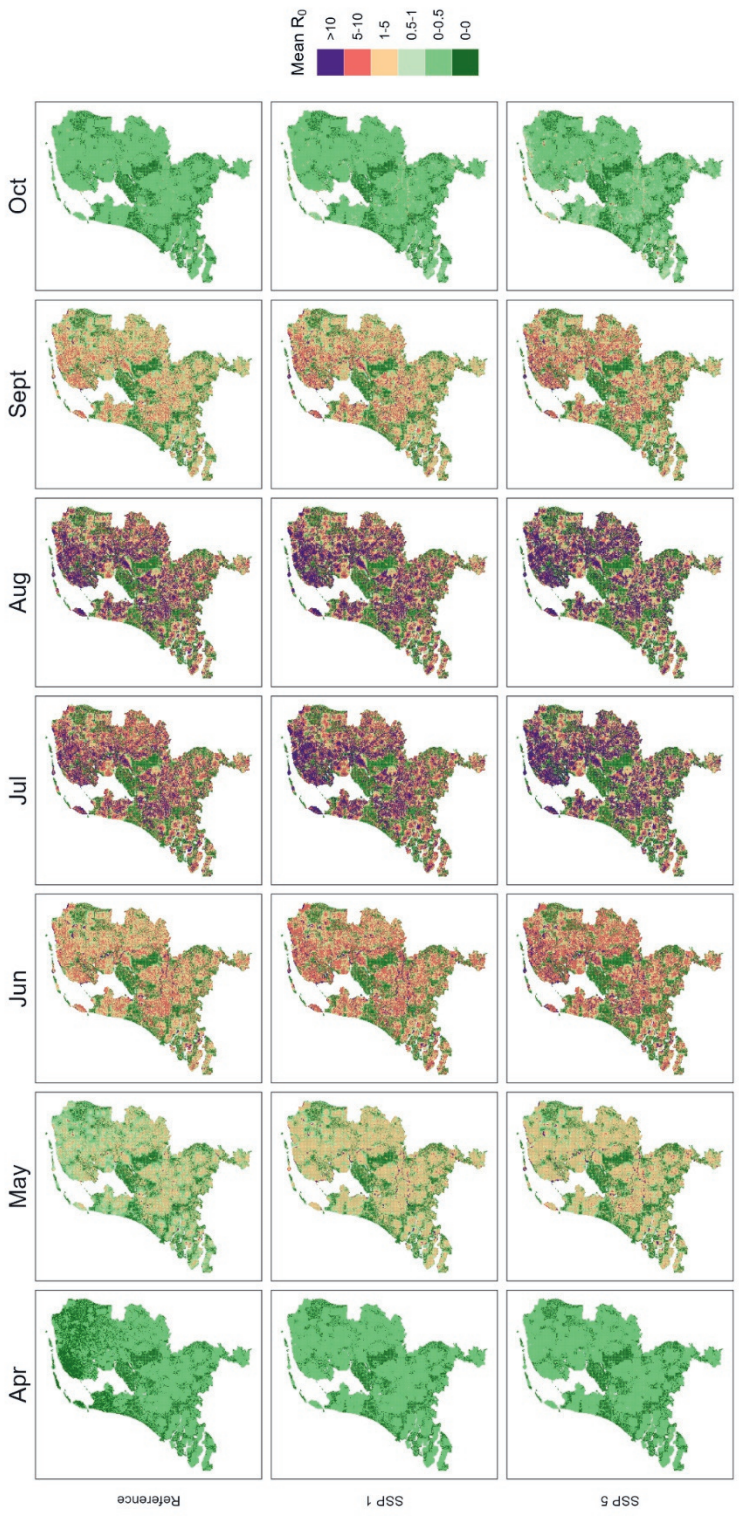


Figure 4. Monthly mean R_0 values for each scenario. Cells without competent hosts are shown as dark green (R_0 set to zero).

4. Discussion

In this study, we investigated the potential for Rift Valley fever virus (RVFV) outbreaks in the Netherlands under one reference and two future Shared Socioeconomic Pathway (SSP) scenarios. Our analysis revealed that the transmission risk and duration of the transmission season increased in both future scenarios.

The potential transmission season of RVFV was longest in the "fossil-fuelled" scenario (SSP5), the scenario in which temperatures were highest and *Ae. vexans* abundance peaks earliest. The SSP5 scenario demonstrated the highest mean R_0 across all locations, indicating a more consistent transmission risk. However, a lower number of grid cells had competent vector(s) and host(s) in SSP5. As this is the first study to investigate the differences of RVFV transmission between different SSP scenarios, we cannot compare our findings with others. For West Nile virus (WNV), another mosquito-borne virus transmitted by (mainly) *Culex* species mosquitoes, Farooq et al. (304) have shown a predicted increase in WNV risk in all future scenarios, especially in Western Europe and for the SSP5 scenario, which is in line with our findings.

Monthly mean R_0 values were highest in areas with high *Ae. vexans* abundance in June (in wetlands and surrounding rivers) and in areas with high livestock densities in July-August. During July and August the abundance of *Cx. pipiens/torrentium,* which is present nationwide, is highest and thus facilitates transmission across the country (200). The number of consecutive days with $R_0>1$ was higher in both future scenarios compared to the reference scenario. As import risk via animal trade is considered to be low, the main risk for introduction comes from imported infectious vectors (283) which may be imported via air or sea transport (16). Two major travel and trade hubs (Schiphol airport and the Rotterdam harbour) are located in areas with a relatively low number of consecutive days with an $R_0>1$, which potentially reduces the probability of establishment, although this is dependent on connectivity to higher risk locations.

One of the main factors that affects our outcomes is the estimation of the vector-to-host ratio, which is closely linked to the input data for hosts and vectors and the host-preference of vectors. The vector-to-host ratio is an important parameter that can significantly influence the R_0 estimates (305,306). In this study, livestock input data was relatively detailed. However, decisions in data handling did have an effect on vector-to-host ratios (removal of veal calves, redistributing sheep from large sheep farms across local regions). Estimating the relative abundance of vectors, especially *Ae. vexans,* was challenging. We used data from a field experiment carried out at a single location in the Netherlands to estimate the number of daily bites per month by both vectors separately. With these estimates we obtained a scaling parameter value for both vector species. The model demonstrated a less satisfactory fit for *Ae. vexans* compared to *Cx. pipiens/torrentium*. This could be due to the fact that a very small

number of observations of *Ae. vexans* landings and bites on cattle and sheep were made during the field experiment, which in turn could be due to a varying number of observations moment per month. This could have led to a lower estimate of the scaling parameter for *Ae. vexans*. Moreover, the scaling parameters used in the model are reliant on host-preferences, for which very little information is available. Additionally, it was difficult to estimate the proportion of total bites on competent hosts. We based this on the assumption that an equal total number of (competent and non-competent) hosts is available in each location, given the wide range of animals these mosquitoes bite on (219,307,308). For the future scenarios, we assumed that the non-competent hosts are reduced in the same percentages as competent hosts. Due to the inherent uncertainty in the vector-to-host ratios, the absolute R_0 values in our results are of limited value. Nevertheless, the relative changes in R_0 across scenarios, such as the length of the transmission season, spatial differences and increased values in future scenarios, are likely to be robust.

Furthermore, it should be noted that the temperature and mosquito input data was averaged over 30-year periods. This allowed us to generate a general approximation of RVFV transmission in future scenarios, but does not capture inter annual variation. Both future scenarios will experience more extreme weather events such as floods, which are now averaged out over 30 years and thus might lead to less extreme results (112). Past outbreaks as well as other modelling studies have shown that transmission risk differs significantly between years (17,295).

One other study has investigated RVFV transmission potential in the Netherlands (298). In contrast to our findings, Fischer et al. (298) found a higher risk of RVFV transmission potential and persistence in areas with low livestock densities, which is largely due to the assumption that no non-competent hosts are present in their model. This results in lower estimated risk of persistence in the north (Friesland), and higher risk in areas with low numbers of sheep and cattle. The use of a different outcome measure complicates comparing our results with their findings. A hazard map for the establishment of RVFV by Esser et al. (285) showed the highest risk in the south (provinces of Zeeland an Limburg) which was likely due to higher estimated temperatures in their input data. Our results also show some areas in the south-west (Zeeland) that have high yearly mean R_0 values, but a strong contrast is observed when comparing the most northern province (Friesland). The method of risk calculation by Esser et al. (285) was based on ranking grid cells based on presence of risk factors for establishment that consisted of abiotic conditions, vector abundance and host availability. The low risk estimate by Esser et al. could be due to their estimates of (ruminant) livestock abundance. However, direct comparison of outcomes is not possible due to the different approaches used.

Further research is needed to elucidate the potential impact of RVFV outbreaks in the Netherlands. While absolute estimates of R_0 for emerging pathogens should be considered with caution and are highly sensitive to model assumptions, the future trends illustrated in this study are robust to a range of assumptions. Sensitivity analyses need to be performed to assess the robustness of the outcomes with regards to changes in parameter values. This is especially important for the vector-to-host ratio, as this was difficult to estimate and likely has a large influence on the R_0 values. Future modelling efforts could incorporate additional components of the model such as differences in flood risk, housing types of animals, wildlife (as potential competent or non-competent hosts) (63), movement of hosts (travel and trade) and transmission by direct contact for each scenario. In this study we have only modelled daily R_0 values, which does not allow for conclusions about the potential extent and severity of outbreaks for humans and animals. Moreover, surveillance (early-detection) and response, as well as the quality and accessibility of health care are different in future scenarios and thus might result in altered probability of a timely response (112).

The results of this study highlight the need for increased preparedness for RVFV outbreaks in the future. It is essential to establish sustainable plans for surveillance, prevention and response. This includes the development of safe and effective vaccines for humans and animals, in order to safeguard their health and welfare, as well as the economy.

Acknowledgements

The authors would like to acknowledge Jaap van Os for his help with acquiring the livestock data and the expert opinion of Reinard Everts for generating the future sheep distributions. Hélène Cecilia, Clara Delecroix, Afonso Dimas Martins are thanked for their modelling advice. Furthermore, Armin Elbers and Jose Gonzales Rojas are acknowledged for their cooperation and use their field experiment data to estimate the relative abundance of mosquitoes.

Supplementary material

Input data and data handling

S.1 Temperature

Daily mean temperatures were obtained from the Royal Netherlands Meteorological Institute (KNMI) (289). For the future scenarios, the ensemble data for the KNMI wet and dry scenario were averaged (Figure S1).

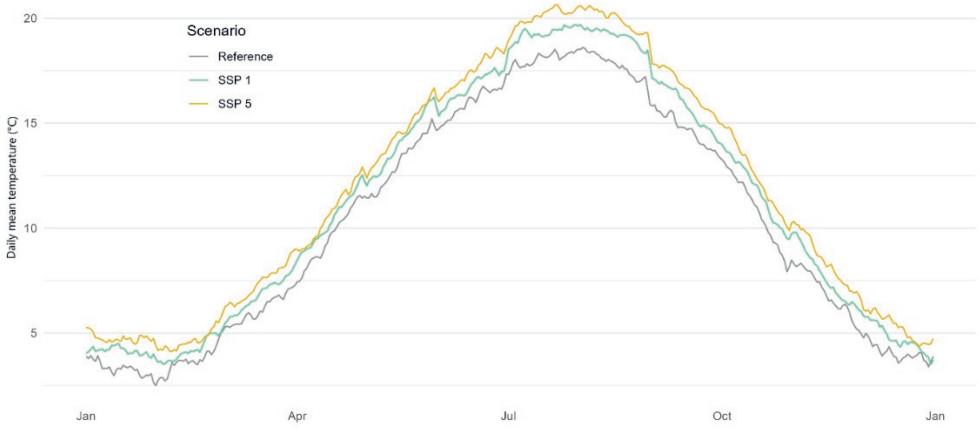


Figure S1. Mean daily temperatures for the reference and future scenarios

S.2 Land use

Current and future land use maps were created by Dellar et al. (303) (Figure S2). An overview of the grid cells per land use type in each scenario is shown in Table S1. The land use maps were used to guide the locations of livestock and relative abundance of mosquitoes in future scenarios as described below.

Table S1. Number of grid cells (%) for per land use type in each scenario. Increase or decrease in future scenarios is shown in green/red as percentages compared to reference. *Non-forest nature

Land use type	Reference (% of total)	SSP1 (% of total)		SSP5 (% of total)	
Urban	5398 (15.2)	5998 (16.9)	↑11%	7453 (21.0)	↑↑38%
Pasture	10551 (29.7)	7516 (21.2)	↓↓29%	9129 (25.7)	↓13%
Crops	14562 (41.0)	14345 (40.4)	=	13623 (38.4)	↓6%
Forest	3021 (8.5)	3602 (10.1)	↑19%	3082 (8.7)	=
NFN*	1970 (5.5)	4041 (11.4)	↑↑↑105%	2215 (6.2)	↑12%

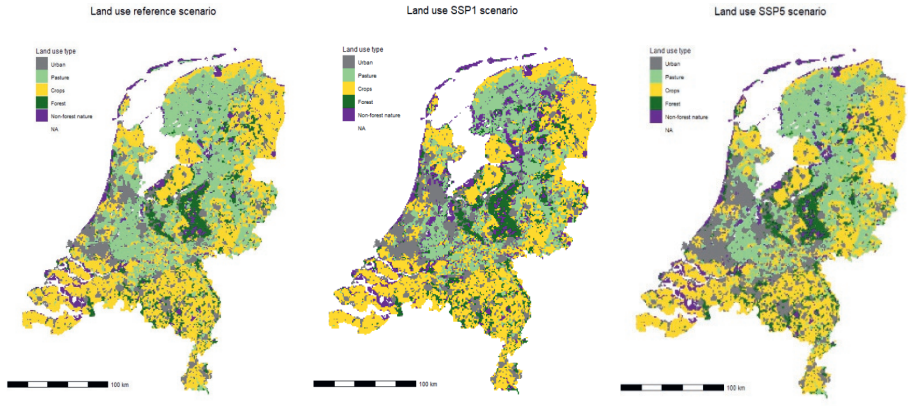


Figure S2. Land use map for each scenario.

S.3 Livestock population

An overview of sheep and cattle numbers per land use type for each scenario are shown in Table S2 and Table S3, respectively. The majority of sheep farms are small farms (<100 sheep). However, large farms may hold up to 5000 sheep. Based on expert opinion, we distributed sheep from large farms (>500 animals) over a 11x11km grid around the grid cell of the farm coordinates. This was done by moving a proportion of sheep to pasture in the surrounding area. A gaussian distribution matrix was used to distribute sheep of large farms over the surrounding (11x11) grid cells. Animals that were located in grids without a land use type (e.g. sea) were removed from the dataset.

Sheep lambing peaks between January and April and usually takes place indoors after which animals are turned out to pasture (R. Everts, personal communication). We assumed a 1:20 male to female ratio and an average of 1.8 lambs per ewe per year. This was implemented by setting the input data at 1st of January and increasing the population with a daily growth rate of 0.00834 (final population/starting population^(1/120)-1) until the 1st of May. Then, we assumed a stable population until the 7th of September after which many animals are sent to slaughter and the population returns to the same level as the 1st of January. We thereby assume a stable population over the years.

Table S2. Sheep numbers and percentages per scenario for each land use type.

Land use type	Sheep reference (%)	Sheep SSP1 (%)	Sheep SSP5 (%)
Urban	114879 (10.0)	147311 (13.7)	116270 (11.6)
Pasture	577892 (50.4)	442710 (41.1)	472674 (47.3)
Crops	400518 (34.9)	388825 (36.1)	338412 (33.9)
Forest	30596 (2.7)	53412 (5.0)	38148 (3.8)
Non-forest nature	22242 (1.9)	45548 (4.2)	33161 (3.3)
Total	1146127	1077806 (-6%)	998665 (-13%)

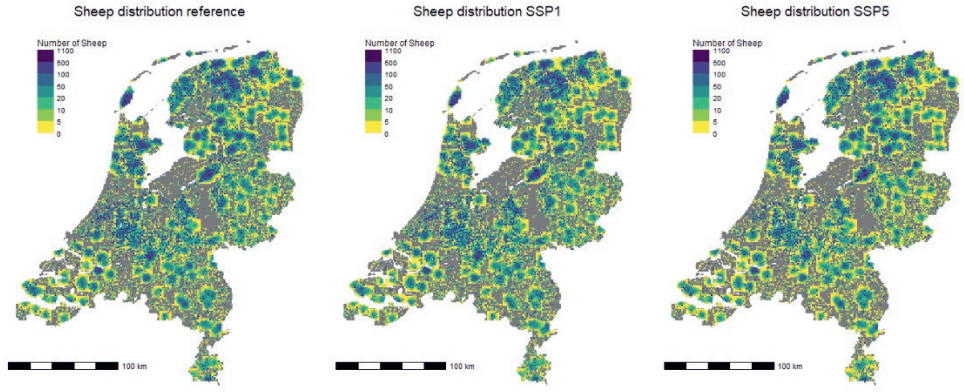


Figure S3. Sheep distribution for each scenario at 1st of January.

Table S3. Cattle numbers and percentages per scenario for each land use type.

Land use type	Cattle reference (%)	Cattle SSP1 (%)	Cattle SSP5 (%)
Urban	76809 (2.8)	38404 (2.8)	0 (0)
Pasture	1608777 (57.6)	804388 (57.6)	1533672 (61.0)
Crops	1047556 (37.5)	523778 (37.5)	980856 (39.0)
Forest	45768 (1.6)	22884 (1.6)	0 (0)
Non-forest nature	15054 (0.5)	7527 (0.5)	0 (0)
Total	2793964	1396982 (-50%)	2514528 (-10%)

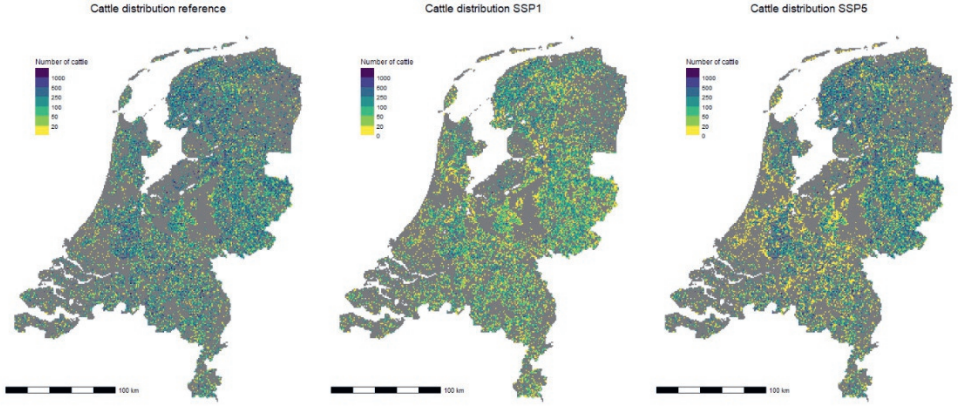


Figure S4. Cattle distribution for each scenario.

S.4 Vector populations

Abundance (on an arbitrary scale) of *Cx. pipiens/torrentium* was obtained from Krol et al. (291) (Figure S5). The probability of occurrence of *Ae. vexans* for all scenarios was obtained from Dellar et al. (292). For both vector species, the ensemble mean of the 1991-2020 model was used for the reference scenario. For the future scenarios, we

averaged ensemble means for the wet and dry KNMI scenarios (2036-2065) for both SSP1 and SSP5.

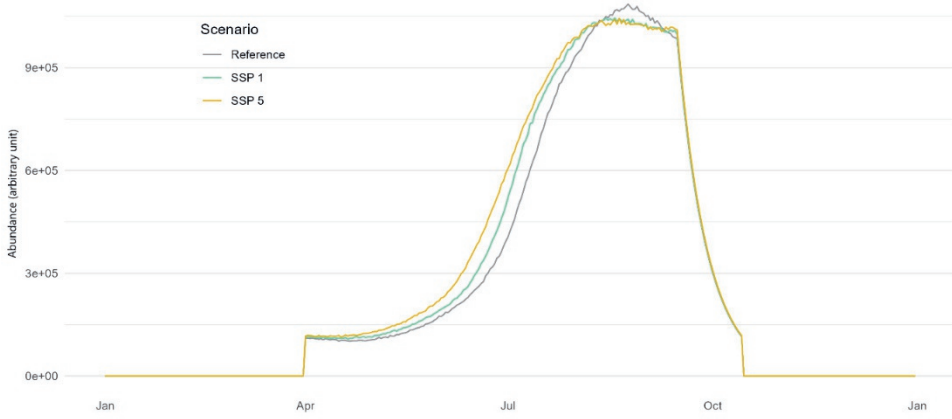


Figure S5. Mean abundance of *Culex pipiens/torrentium* in all scenarios.

S.4.1 Aedes vexans probability of occurrence to arbitrary abundance

The available probability of occurrence maps for *Ae. vexans* needed to be transformed to relative abundance estimates, for all scenarios. For that purpose, we trained a small generalised additive model (GAM) in mgcv, using a dataset of 183 trapping efforts where *Ae. vexans* was caught, and 1136 where *Ae. vexans* was not caught. This dataset is the same dataset used by Dellar et al. (292), but also included number of mosquitoes per catch (data not shown). The GAM model was trained to predict the observed relative abundances, using the following predictor variables: (1) presence/absence maps (292), (2) precipitation and temperature from a 30-year climate normal from 1991-2020 (289), and (3) land use type, and a negative binomial function to link the predictions to the observations. The model is described in the following terms:

$$abundance \sim s(presence, landuse, bs = \text{fs}, k = 5) + s(temp, k = 5) + s(temp_{prev}, k = 5) + s(precip, k = 5) + s(precip_{prev}, k = 5)$$

That is, abundance is predicted by the fitted non-linear relationships of presence per land use type, daily temperature normal, daily temperature normal of 30 days prior, precipitation normal, and daily precipitation normal of 30 days prior. Here, “normal” refers to a 30-year baseline, e.g. for the reference scenario that period covers the average daily temperatures in the time period 1991-2020. For the future scenarios, these cover the predicted average daily temperatures for the period of 2036-2065.

No additional reduction penalties were applied on the non-linear relationships other than setting the number of knots to 5, and using the default tp smooths and a factor-smooth interaction when fitting the importance of presence per land use type. The

quality of the model was tested using DHARMa, with no substantial problems found (one outlier in the data, one mildly significant residual pattern in the upper quantile for temperature). The response curves of the GAM model are shown in Figure S6, and the results of the model are shown in Figures S7a and S7b.

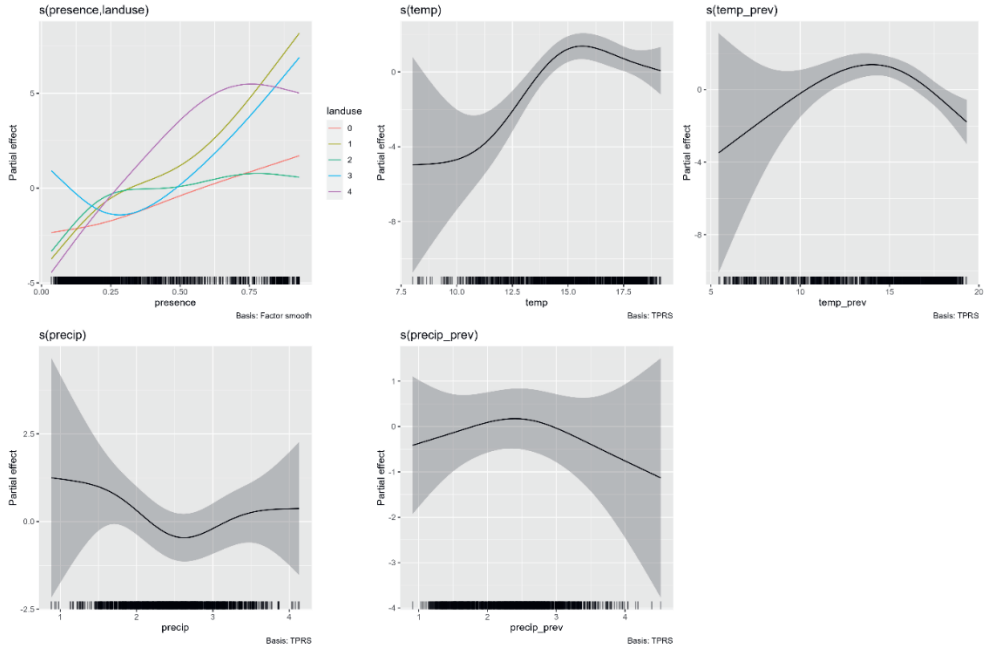


Figure S6. GAM response curves. Top-left plot shows the relationship between probability of presence and contribution to abundance per land use type, when correcting for temperature and precipitation and temperature and precipitation 30 days before. Land use legend is (0) urban, (1) pasture, (2) cropland, (3) forest, (4) non-forest nature, as per (303).

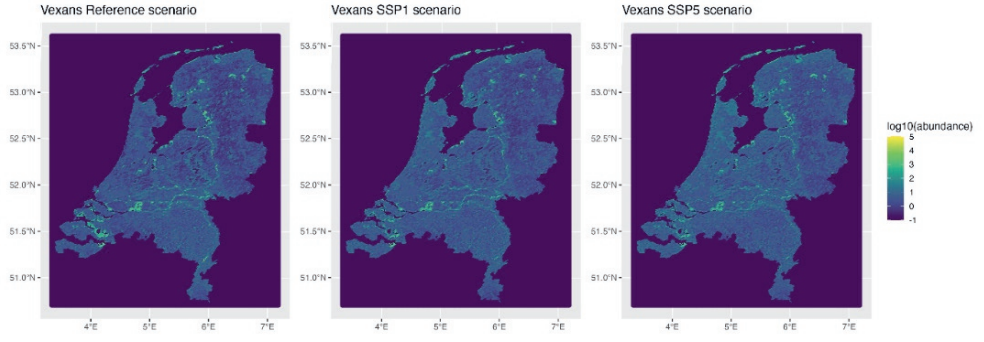


Figure S7a. Abundance (arbitrary unit) of *Ae. vexans* for each scenario.

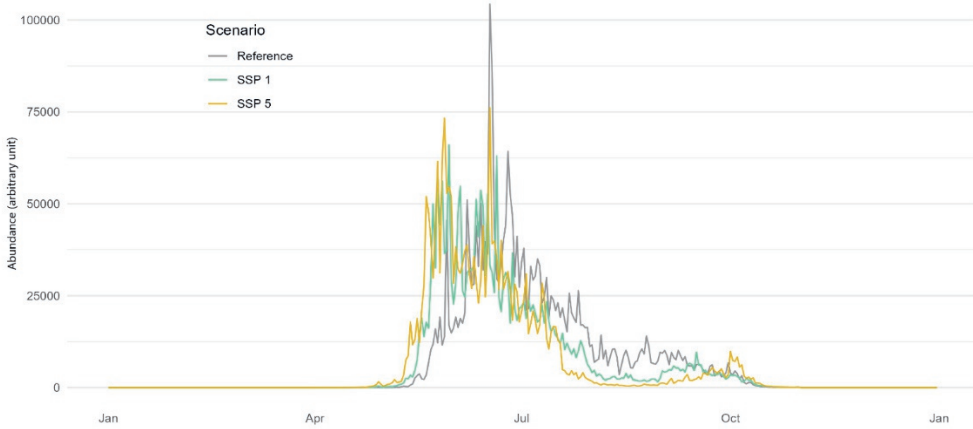


Figure S7b. Mean abundance of *Aedes vexans* for all scenarios over time.

S.5 Scaling the vector-to-host ratio

As the exact number of mosquitoes per grid cell is not available from data, a scaling parameter was calculated for each vector species in order to achieve the vector-to-host ratio. This scaling parameter was estimated based on a field experiment as described executed by Elbers et al. (in preparation). In this field experiment, mosquitoes were aspirated from a cow and sheep for 5 minutes per hour on a farm in the Netherlands throughout one vector season. From this data, we calculated average daily number of bites for *Cx. pipiens/torrentium* and *Ae. vexans* for each month and host type. With these values, we estimated a scaling parameter. The number of cattle and sheep, biting rates, proportion of bites on competent hosts and the relative number of mosquitoes per species were taken from the input data for the grid cell where the experiment was carried out. The average numbers of hosts (N_{hc} *and* N_{hs}), relative abundance of vectors (N_i), biting rates (α_i) and proportion of bites on hosts (ν_i) were calculated per month for the months June, July and August.

$$Bites\ on\ cattle = (N_i * scaling\ value)/(N_{hs} + N_{hc}) * \alpha_i * \nu_i * \theta_{ic} \quad \text{(S1)}$$

$$Bites\ on\ sheep = (N_i * scaling\ value)/(N_{hs} + N_{hc}) * \alpha_i * \nu_i * \theta_{is} \quad \text{(S2)}$$

Using equations S1 and S2, we estimated the scaling value by minimising the sum of squared errors between observed and predicted number of bites on cattle and sheep per month. The scaling factor of *Cx. pipiens/torrentium* was used as multiplication factor for mosquito abundance input data to transform the arbitrary units into realistic vector-to-host ratios. The fit for the *Ae. vexans* scaling factor was not as robust as that for *Cx. pipiens/torrentium*, as very limited counts were observed in the field experiment (and *Ae. vexans* peaks at specific points of the year in specific locations (Figure S8).

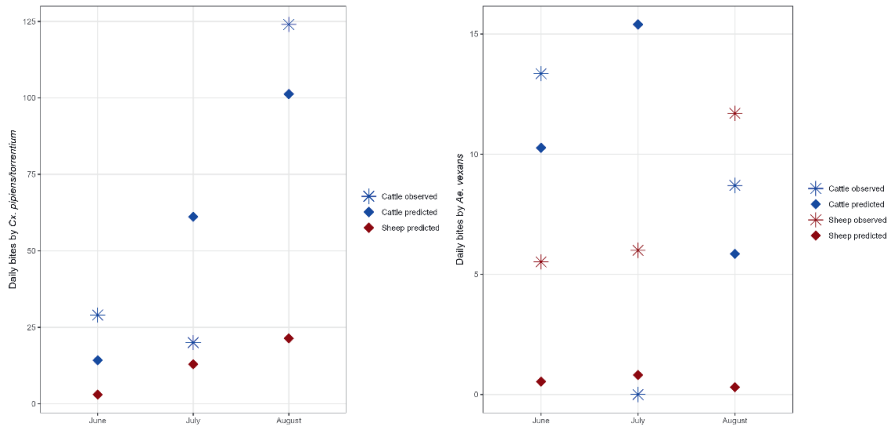


Figure S8. Fit of the scaling model compared to the observed field experiment data for *Cx. pipiens/torrentium* (left) and *Ae. vexans* (right) for the grid cell where te field experiment was carried out.

Parameterization

S.6 Transmission probabilities

Transmission probabilities were assumed to be independent of temperature. A recent literature review by Drouin et al. (286) shows an overview of the vector competence of multiple mosquito species for RVFV based on previous (laboratory) studies. We used the overall dissemination rates (%) of 13.6 for *Ae. vexans* and 13.5 of *Cx. pipiens/torrentium* which results in host to vector transmission probabilities of 0.14 for both species. For the vector to host transmission probabilities, we used the transmission rates among mosquitoes having a disseminated infection (%). These were 38.3 for *Ae. vexans* and 93.6 for *Cx. pipiens*, resulting in transmission probabilities of 0.38 and 0.94, respectively.

S.7 Temperature dependent parameters

As the extrinsic incubation period, biting rate and mortality rate of mosquitoes are assumed to be temperature dependent. These parameters were calculated daily for each grid cell for all three scenarios.

Biting rates

Cx. pipiens/torrentium biting rates were based on Rubel et al. (293). This formula does not result in negative biting rates at temperatures lower than 10 degrees Celsius in contrast to other formulas like that of Shocket et al. (297) (Figure S9). The *Ae. vexans* biting rate function results in values below 0 in case temperatures are low (<9.6 degrees Celsius), all values below 0 were set to 0 (Figure S10).

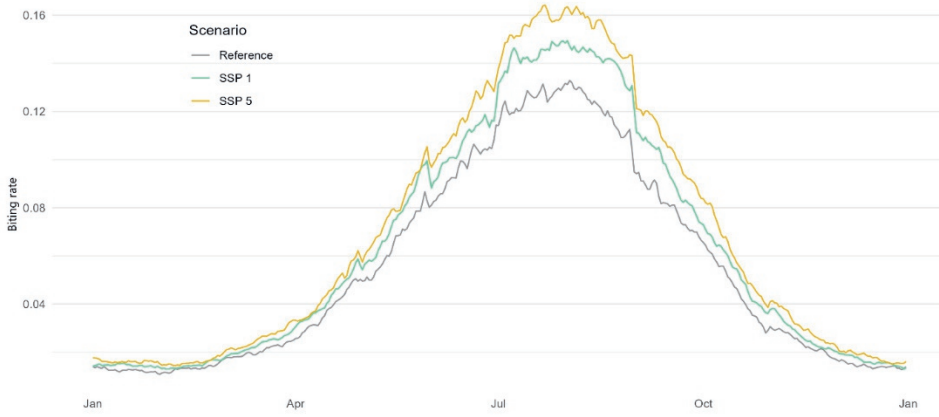


Figure S9. Mean daily biting rates of *Culex pipiens/torrentium* in all scenarios over all grid cells.

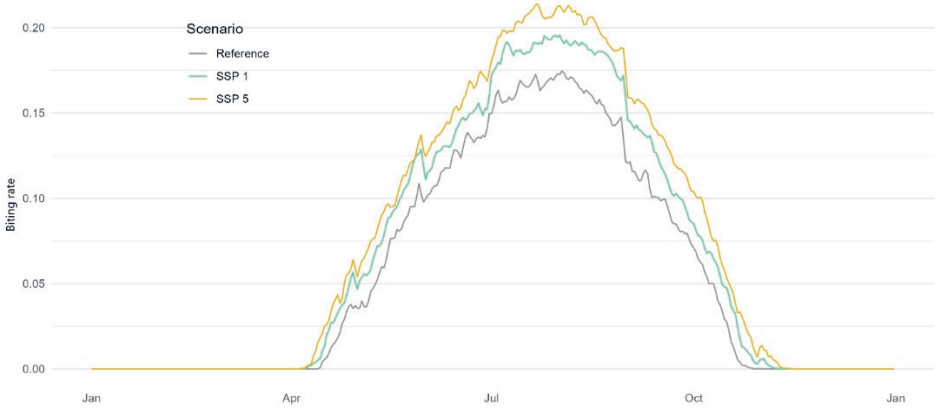


Figure S10. Mean daily biting rates for *Aedes vexans* in all scenarios over all grid cells.

A parameter representing the proportion of total bites from vectors to competent hosts (cattle and sheep) was implemented based on the assumption that a proportion of bites will be on non-competent hosts. In cells having relatively large numbers of competent hosts, all bites are assumed to be on competent hosts. For lower numbers of competent hosts, we assume *Cx. pipiens/torrentium* to be ornithophilic and *Ae. vexans* to be mammophilic (287,288). For *Cx. pipiens/torrentium*, we calculated the 99% percentile of competent hosts for each day in cells with at least one competent host, (which then receive 100% of bites) and scaled down from the 99% percentile. For *Ae. vexans* we scaled from the 50% percentile of cells with at least one competent host, resulting in more cells where 100% of bites are on competent hosts.

Additionally, a relative preference for cattle and sheep was incorporated separately for both vector species using the following equation, which was based on Cecilia et al.

(295). This proportion of blood meals of vector *i* taken on host population *j* was calculated as follows:

$$\theta_{ij} = \left(\frac{\emptyset_{ij} * N_j}{\emptyset_{is} * N_{hs} + \emptyset_{ic} * N_{hc}} \right)$$

Where $\emptyset_{ij}$ is the relative preference of one of the vector species for one of the host species and N_{hs} and N_{hc} are the total number of hosts for that host species in the grid cell.

Mortality rates

Mortality rates of *Cx. pipiens/torrentium* were calculated based on a formula by Shocket et al. (297), which states a maximum mortality rate of 0.17 for temperatures higher than 31 degrees Celsius. From the 15th of September, the mortality rate was changed into a diapause rate for *Cx. pipiens/torrentium* to simulate diapause and removal of host-seeking mosquitoes from the population. This starting date of the diapause period was based on data from Field et al. (309) and Blom et al. (310). Daily mosquito counts were then calculated by subtracting the mosquito count of the previous day times the diapause rate (0.071429) from the mosquito count of the previous day. For *Ae. vexans* the mortality rate was based on a function by Fischer et al. (298), which was based on a laboratory study on *Ae. vexans* mosquitoes by Costello and Brust (311) (Figure S11). Infection with RVFV was not assumed to have an effect on mosquito mortality. For *Cx. pipiens*, only one study exists that suggests increased mortality in mosquitoes infected with RVFV (312). For *Ae. vexans* these studies do not exist.

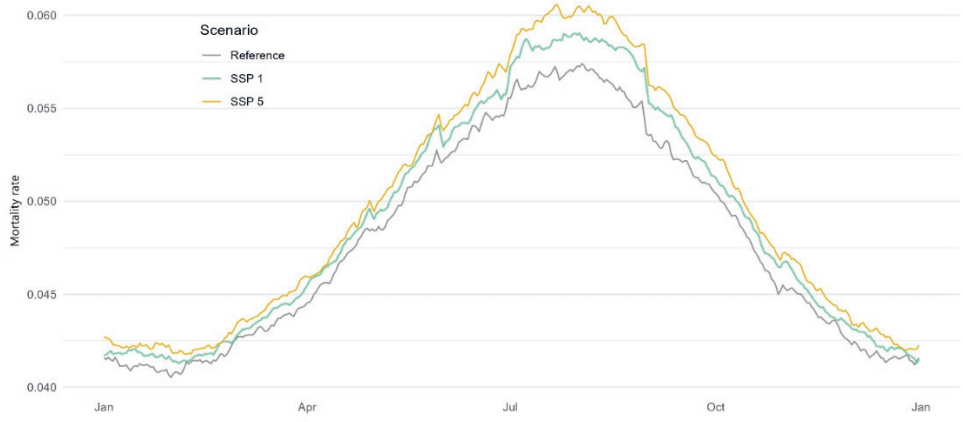


Figure S11. Mortality rates for *Aedes vexans* in all scenarios.

Extrinsic incubation rates

The incubation rates for *Ae. vexans* were based on a formula by Shocket et al. (297) for *Ae. taeniorhynchus*, as no published function was available for *Ae. vexans* (Figure S12). For *Cx. pipiens/torrentium,* the incubation rates were based on *Cx. pipiens/torrentium* infected with West Nile virus as described in Shocket et al. (297) (Figure S13) as no data was available for infections with RVFV.

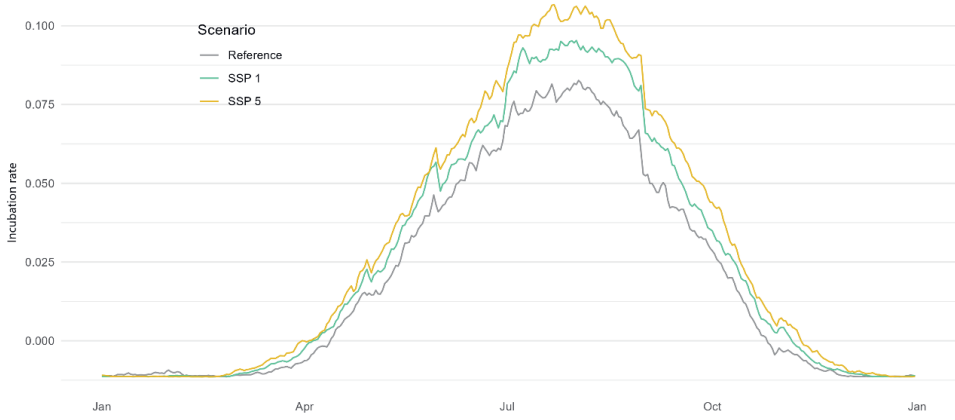


Figure S12. Daily mean incubation rates for *Aedes vexans* in all scenarios over all grid cells.

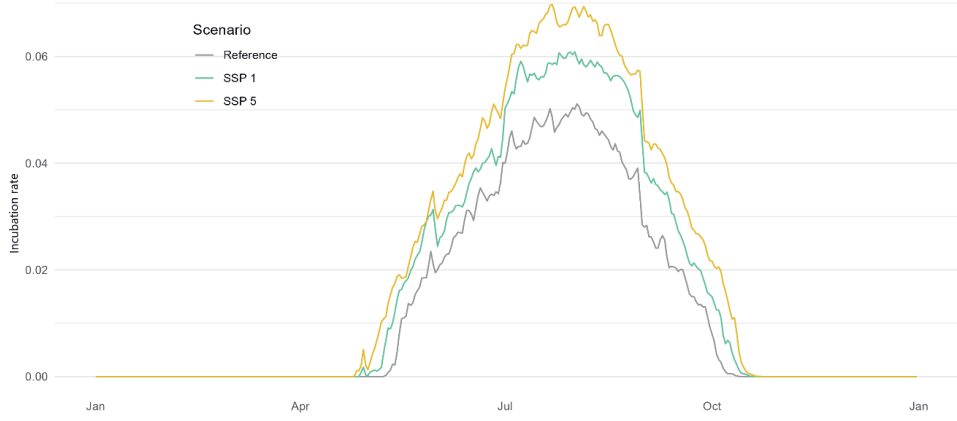


Figure S13. Daily mean incubation rates for *Culex pipiens/torrentium* in all scenarios over all grid cells.

S.8 Transmission matrix

0	0	0	0	0	0	$\alpha_p * \nu_p * \theta_{pc} * \rho_{pc}$	$\alpha_v * \nu_v * \theta_{vc} * \rho_{vc}$
0	0	0	0	0	0	$\alpha_p * \nu_p * \theta_{ps} * \rho_{ps}$	$\alpha_v * \nu_v * \theta_{vs} * \rho_{vs}$
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	$\alpha_p * \nu_p * \theta_{pc} * \rho_{cp} * \frac{N_{mp}}{N_{hc}}$	0	$\alpha_p * \nu_p * \theta_{ps} * \rho_{sp} * \frac{N_{mp}}{N_{hs}}$	0	0	0
0	0	$\alpha_v * \nu_v * \theta_{vc} * \rho_{cv} * \frac{N_{mv}}{N_{hc}}$	0	$\alpha_v * \nu_v * \theta_{vs} * \rho_{sv} * \frac{N_{mv}}{N_{hs}}$	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

S.9 Transition matrix

$-(\mu_{hc} + \varepsilon_{hc})$	0	0	0	0	0	0	0
0	$-(\mu_{hs} + \varepsilon_{hs})$	0	0	0	0	0	0
ε_{hc}	0	$-(\mu_{hc} + \delta_c + \gamma_c)$	0	0	0	0	0
0	ε_{hs}	0	$-(\mu_{hs} + \delta_s + \gamma_s)$	0	0	0	0
0	0	0	0	$-(\mu_{mp} + \varepsilon_{mp})$	0	0	0
0	0	0	0	0	$-(\mu_{mv} + \varepsilon_{mv})$	0	0
0	0	0	0	ε_{mp}	0	$-\mu_{mp}$	0
0	0	0	0	0	ε_{mv}	0	$-\mu_{mv}$

Chapter 8.

General discussion

The overall objective of this thesis is to provide insight in the epidemiological situation, (response) surveillance options and potential risk of mosquito-borne viruses (MBVs) in animals in the Netherlands, in order to inform future research and surveillance strategies. This was done by executing multiple cross-sectional surveys, a longitudinal survey, questionnaires, interviews as well as statistical and mathematical modelling approaches.

In this discussion, I will focus on how this thesis has filled knowledge gaps about MBV circulation and possibilities for research and surveillance with a focus on the Netherlands in different animal species. Furthermore, I will discuss the use and interpretation of serological methods, implementing One Health arbovirus research and surveillance in practice, and raise ethical questions related to MBV surveillance in animals. I will broaden the scope to put this thesis into context of the One Health PACT (OHPACT) research consortium. Finally, I will sum up the conclusions and provide recommendations and an outlook for the future.

8.1 Epidemiological insights

Usutu virus (USUV) caused a large outbreak in 2016 in the Netherlands that resulted in bird die-offs of mainly blackbirds (*Turdus merula*), and evidence of circulation has been found in wild birds, humans and mosquitoes in the subsequent years (55,56,123). West Nile virus (WNV) was first detected in mosquitoes and a bird in 2020 (58). In the same year, human cases of WNV were retrospectively detected in two regions (57,125). Funding of testing suspected cases in horses (*Equus caballus*) was then implemented to lower the (financial) barriers for reporting, in order to detect WNV circulation early (313). In 2021 and 2022, 33 and 66 serum samples of horses with unexplained neurological signs were submitted. None of these could be confirmed as WNV infections (313). No human or equine cases were observed in 2021 and 2022, which may create the impression that the virus was not circulating. However, in **chapter 5**, we describe circulation of WNV in both areas in 2021 in petting zoo and backyard chickens (*Gallus gallus domesticus*), which remained undetected by other surveillance methods. Additionally, in **chapter 4** we found evidence of WNV infections in wild boars (*Sus scrofa*) already in 2018 and 2019, suggesting undetected introduction(s) before the first official detection in 2020. In October 2022, one WNV positive bird was sampled in a new region (126). The syndrome surveillance system resulted in one clinical WNV equine case detection in 2023, but the location of infection remained unclear as the horse originated from Germany (314).

As the baseline seroprevalence of WNV and USUV was still unknown, cross-sectional serological studies in several animal species were carried out and described in this thesis. The results of **chapter 2** show us that the seroprevalence of USUV and WNV is very low in dogs (*Canis lupus familiaris*) and horses in the Netherlands. We found an

observed seroprevalence of 0.27% for WNV in horses (0.0% in dogs) and 0.39% for USUV in dogs (0.0% in horses). In the neighbouring country Germany, repeated seroprevalence studies between 2010 and 2022 of WNV in horses found prevalences between 0% and 13.77% in (107,149,315,316). These seroprevalences were dependent on the period and region of sampling, as some studies were risk-based (sampling animals in regions where an outbreak had occurred previously). It must be noted that all of these studies mention a proportion of the seropositive horses had (potentially) been abroad and/or vaccinated. A serosurvey in southern France in 2016 revealed higher USUV and WNV seroprevalences in 235 horses of 3.85% and 13.19%, respectively. The high WNV seroprevalence in horses was most likely due to a WNV outbreak in the area in 2015 as all horses were unvaccinated (74). The same study reports that a serosurvey in the same region revealed lower seroprevalences of 1.08% for USUV and 0.54% for WNV in (pet) dogs 2019-2020 (74), which is similar to our results as similar screening and confirmation methods were used. In **chapter 4**, we investigated seropositivity of wild boars in the Netherlands from 2018 to 2021. Based on the findings in chapter 2 and published literature, we expected slightly higher prevalences in wild boars, as vector exposure is suggested to be higher in this species (109). However, we found much higher proportions of wild boar with neutralizing antibodies against WNV and/or USUV, with a declining trend that was observed over the four-year period. For USUV this was in line with in the observed trends in Dutch blackbirds (192). We also found that in 2018, many of the USUV infected wild boars are classified as equivocal, due to cross-reactive WNV titres, if interpreted by the classical method of interpretation. The probability of WNV infection within the ELISA positive wild boars increased over the period 2018-2021, which is in line with the expansion of WNV circulation over these years as also described in **chapter 5** for 2021 (58,188,207). In that study, seroprevalence and flock prevalence of WNV increased after May 2021, indicating circulation that year. Comparing seroprevalences of **chapter 4** and **chapter 5** is hampered by the use of different serological assays and interpretation methods. Nevertheless, both studies have shown the possibility of detecting (local) WNV circulation in absence of human and equine cases.

Furthermore, while it was not the focus of **chapter 4**, we found multiple wild boars with high Tick-Borne Encephalitis virus (TBEV) neutralizing titres in VNT. we found indications for a declining trend in TBEV positives from 6.2% in 2018, to 5.3%, 4.3% and 2.6% in 2019-2021, respectively. This is remarkable as an increase in human TBEV has been observed during those years (142). It is also in contrast with studies from Belgium that reported an increasing seroprevalence of TBEV in wild boars; from 4.2% in 2013 to 9.27% in 2019-2020 (131,317). Interestingly, It should be noted that these percentages could be an underestimation of the results, as the sensitivity of the used screening assay (competitive ELISA) for TBEV is suggested to be low (318). Therefore, further

research using a more sensitive screening test for TBEV is needed to investigate the TBEV seroprevalence in wild boars in the Netherlands.

Sindbis virus (SINV) was not known to circulate in the Netherlands, but had already been detected in many European countries, including Germany (163). Two previous studies did not detect SINV in Dutch mosquitoes (169,170). **Chapter 3** describes the first seropositive horses and wild birds, as well as one PCR positive wild bird. The seroprevalence of 0.82% in horses found is lower than the 3.5% (6/171) found in Swedish horses (106). This is not remarkable as SINV has been known to circulate in Sweden for years (167,319). For many countries, the seroprevalence of SINV is still largely unknown. No human cases of SINV have been detected outside of Sweden and Finland in Europe so far. Even though all bird ringers in our study were seronegative, we cannot exclude the possibility that humans in the Netherlands have been infected, potentially without symptoms or without being diagnosed, as awareness among healthcare professionals is probably low.

Most of the MBVs currently present in the Netherlands do not have a major impact on animal health. However, it is expected that due to changes in the drivers of emergence, new viruses will be introduced in the coming decades. In **chapter 7** we modelled the outbreak potential of Rift Valley Fever virus (RVFV) in the Netherlands in a reference and two future scenarios. The future scenarios are based on Shared Socio-Economic Pathways (SSPs), that were transformed to One Health SSPs for the Netherlands by Dellar et al. (112). SSP1 which represents a “nature inclusive” scenario and SSP5 which is a “fossil-fuelled” scenario (112). Results indicate that the transmission season is prolonged in both future scenarios and is longest in SSP5. Higher R_0 values are first observed around river systems and wetlands in June, when *Ae. vexans* populations are high. Two possible RVFV introduction locations, Schiphol Airport and the Rotterdam harbour are located in areas with lower transmission risk and shorter periods during which transmission can occur. Many drivers of MBV emergence are increased in SSP5; an increase in temperature and extreme weather events, huge growth in travel and trade, as well as land use and agricultural changes (112). These changes will increase the risk of RVFV import of infected livestock or vectors through travel and trade (16,320).

All these findings aid in better understanding of exposure and transmission dynamics of MBVs in the Netherlands. This information may be used to guide development of sustainable surveillance systems to enhance preparedness.

8.2 Different animal species in current and future research & surveillance

Based on the findings in this thesis, suitability of animal species for MBV research and surveillance varies. This suitability depends, as mentioned in **chapter 1**, on many factors such as; the presence and abundance of the animal species, access to samples or options for active sampling, goal of the surveillance system, role in the transmission cycle, and host-feeding behaviour of vectors. Many previous studies have mentioned different sentinel species as the optimal way for early-detection (chapter 1). As the factors mentioned are different in every setting, one cannot easily compare the suitability of animal species between, or even within, countries. The idea behind the use of (sentinel) animal species in MBV surveillance is based on the ability to detect infection before human cases appear (Figure 1). This would allow for a rapid response to prevent human disease and economic impact.

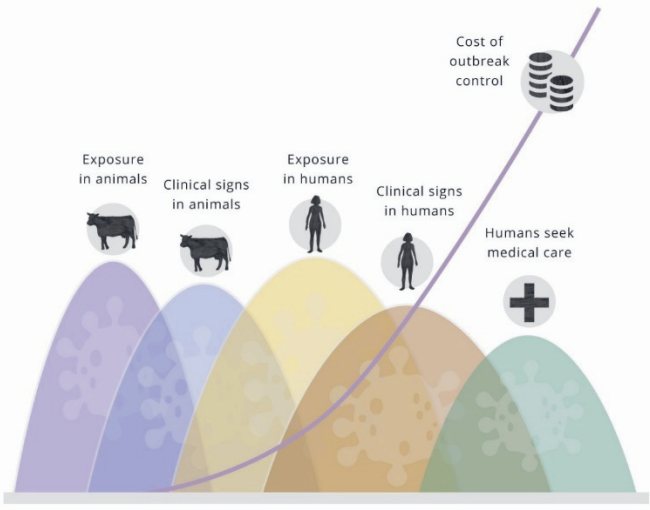


Figure 1. Early detection of zoonotic diseases in animals to prevent higher costs of outbreak control and human disease. (Figure adapted from (321))

Chickens could be a promising sentinel species in the Netherlands for the detection circulation of USUV and WNV, and potentially other MBVs, in absence of human and equine cases (**chapter 5**). Multiple countries such as Greece, Serbia and United States have showed similar results, even though human cases may also appear before seroconversion in chickens is observed (209,322,323). The sensitivity of the sentinel chicken system depends on many factors like the sampling interval (often weekly or biweekly), the number of chickens per flock (ranges from 2-30) and flock placement (76). Sampling of chickens on a (bi-)weekly basis is laborious and cost intensive, and diagnostic tests should be capable of operating with small volumes. Despite these downsides, our findings indicate that petting zoo and backyard chickens are good

sentinels for USUV and WNV surveillance (**chapter 5**). Especially as these animals were mostly located in urban or semi-urban areas, they could point towards potential risks for public health. Additional advantages are the fact that no new chicken flocks have to be bought/placed, as well as the fact that petting zoos are (according to my experience) very willing to participate. Further research on the sampling interval and cost-effectivity is needed to quantify the effectiveness of backyard and petting zoo chickens for use in MBV surveillance. Furthermore, investigation of mosquito species and their blood-feeding patterns in petting zoos is necessary, as we only collected mosquitoes via aspirators after visual inspection (which resulted in low numbers of mosquitoes caught) and not by placing mosquito traps targeting blood-fed females.

The Netherlands hosts a substantial poultry industry, and it has been proposed to utilize commercial laying hens for MBV surveillance. As we found a significant stream of poultry samples in **chapter 6**, this could be a promising approach. Key considerations in establishing such a system include: (1) the extent of exposure of commercial poultry to competent mosquitoes and relevant viruses; (2) the impact of the local vector-host ratio on the system's sensitivity; and (3) whether seropositivity in laying hen farms offers insights into human health risks (Figure 1). With regards to the first consideration, it is hypothesized that free-range poultry are more frequently exposed to competent mosquitoes compared to indoor housed poultry. However, there is currently no literature available to support this statement. Therefore, I performed a pilot study in two closed and one free-range laying hen farms in September 2020. For this pilot, CO_2 baited BioGents BG-Pro traps (Biogents, Regensburg, Germany) were placed inside and outside the barns for four consecutive days. Additionally, mosquito resting traps were placed indoors. Results of this pilot study are shown in Figure 2. Four blood fed *Culex pipiens/torrentium* mosquitoes were caught in total (two in each of the closed farms). The average ratio of indoor/outdoor caught mosquitoes was highest in the free-range farm, but did not differ significantly from the closed farms (Figure 2). It is thus confirmed that competent vectors enter “closed” as well as free-range laying hen farms. More research is needed to investigate the other two considerations, and further elucidate a possible difference between closed and free-range farms. With regards to consideration 3 (insight to human health risk), one could argue that the spatial patterns of poultry farms throughout the Netherlands is biased, as farms are clustered in specific regions of the country (324). Currently, the Dutch poultry sector is still hesitant due to insecurity about economic consequences in case seropositive animals would be detected. As shown in **chapter 6**, establishment of collaborations and networks between stakeholders are needed to lower barriers and hesitancy related to sharing of samples.

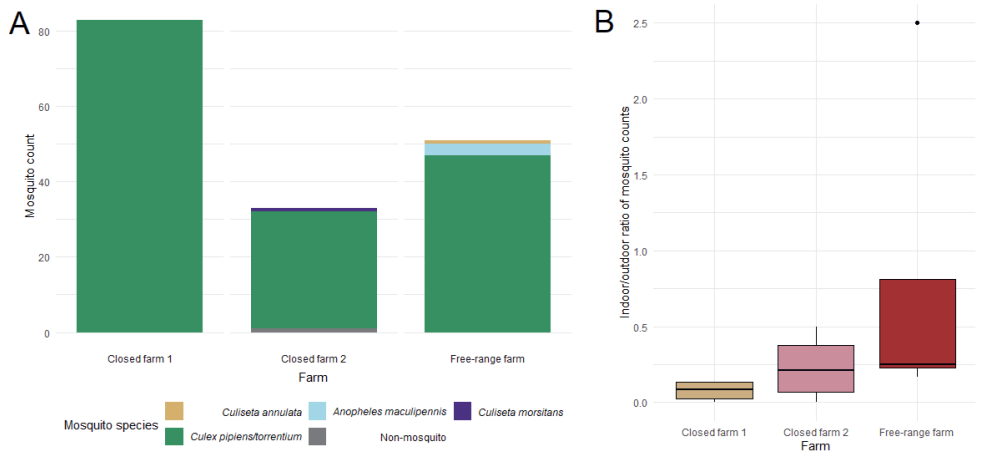


Figure 2. Results of the pilot study in poultry farms. (A) Total mosquito counts per species for three poultry farms. (B) Indoor/outdoor ratio of mosquitoes caught in pilot study with three laying hen farms.

The low seroprevalences of MBVs in horses allow for trend observations over longer time periods as well as detection of clinical cases as a large proportion of the population is still susceptible. Low seroprevalences might be due to low exposure to mosquitoes in relation to housing conditions or the presence of other hosts such as (wild) birds. Considering the known circulation of USUV and WNV in the Netherlands and results of **chapter 4** and **5**, but the low seroprevalence in horses, they might not be the most suitable species to target for early-detection by serology. Furthermore, even though we identified a large stream of diagnostic samples for horses (**chapter 6**), missing metadata such as vaccination and travel history hamper their use in MBV surveillance.

In literature, equine WNV infections are suggested to occur before human cases appear (107,152), comparable to what is shown in Figure 1. However, the predictive value of equine infections for human disease is very country- or even region dependent. In some regions, human cases are not preceded by equine infections and in other regions, only equine infections are detected without any human cases in the same area during that transmission season (322,325,326). Interestingly, we did observe SINV seropositivity (and one potential clinical case) in horses without human case detection (**chapter 3**), in contrast to what had been observed for WNV in 2020 and before. The absence of clinical WNV infections in horses could be due to a high WNV vaccination rate or low exposure to vectors, but current data on these topics are unavailable as our serosurvey was focused on non-vaccinated horses. **Chapter 2** did reveal many horse owners are aware of vaccination (85%), but a smaller proportion actually considers vaccination or had already vaccinated their horse(s) (50.7%). The discrepancy between

detection of cases in horses versus humans is, however, most probably related to the awareness and surveillance efforts. Horse owner knowledge is high (**chapter 2**), so we could expect WNV cases to be recognized by owners. Nevertheless, we noticed a hesitancy from owners and veterinarians to notify suspected cases (personal communication). Two main reasons for this hesitancy are (1) fear and uncertainty about the response from the Netherlands Food and Consumer Product Safety Authority (NVWA) to detection of a case and (2) the lack of treatment options. The lack of transparency about response procedures has also been mentioned for other diseases such as Avian Influenza and African Swine Fever (327,328). The lack of treatment options applies to multiple MBVs, potentially resulting in missed diagnoses of other MBVs as well. Additionally, costs for diagnostic tests have to be paid by the owner when suspected cases are not officially notified, which is another barrier for diagnosing possible cases (personal communication).

Low seroprevalences were also observed for dogs. Dutch pet dogs are often housed indoors and repellents are frequently used (**chapter 2**), potentially lowering exposure to vectors. This is in agreement with a recent study from Namibia that found lower seroprevalences in dogs compared to horses, and proposed vector abundance, host-preference and housing conditions as possible explanations (329). For dogs that spend more time abroad (i.e. military or hunting dogs), it is suggested that seropositivity in dogs might precede detection of human cases (95–98). The Netherlands does not have an extensive system of military dogs that spend a lot of time outdoors and animals may travel abroad, making their use limited for MBV surveillance. Use of commercial diagnostic sample streams is, just like in horses, hampered by unknown travel history and other metadata. For dogs, many samples are shipped to Germany as large laboratories test and store their samples there, and are therefore also difficult to recover and test for other pathogens (**chapter 6**). Repeated sampling of these species is challenging, as this would require different sampling strategies and additional ethical approvals. Nevertheless, a small proportion of dogs may show clinical signs due to infections with WNV or TBEV, thus awareness and testing of suspected cases could help to detect circulation (330–334).

In contrast to horses and dogs, a large proportion of wild boars showed neutralizing antibodies against WNV, USUV and/or TBEV. As samples are already collected and animals are often young (<3 years), this species might be a very suitable indicator species for surveillance of arboviruses. Studies from Italy and France, among others, have suggested the use of boars due to their high exposure to vectors and strong antibody response (109,335). Symptomatic surveillance and repeated sampling are not possible in these animals, and their geographical spread is quite limited in the Netherlands. Wild boar serum samples are often of low quality, which is often related to the collection method, shipment and number of free-thaw cycles (336,337).

Haemolysis and autolysis can affect diagnostic results and interpretation, especially in neutralization assays (185). These factors limit their useability, but as shown in **chapter 4** and in literature, infections in wild boars may occur earlier than in domestic animals and/or humans, which emphasizes their potential value (109). For future studies, other samples collection methods, such as intracavernous venipuncture, could be used to obtain less haemolysed sera (336). The detection of TBEV and WNV infected boars highlights the need to inform hunters and other high-risk groups about use of preventative measures and vaccination.

The Netherlands has a very high livestock density. It would be interesting to investigate the use of domestic ruminants, pigs and poultry for zoonotic arbovirus surveillance. Livestock species can be clinically affected by, and be a risk for infection of humans, for viruses such as Rift Valley Fever virus (RVFV) and Japanese encephalitis virus (JEV), and TBEV. Syndromic surveillance should be in place for RVFV and JEV, as these pathogens could have a large impact on animal health. TBEV testing in (small) ruminants is a promising approach to not only prevent animals disease but also serve as a potential public health benefit since raw milk products from such animals can be infectious and may cause disease in humans (338,339). Furthermore, surveillance of other orthoflaviviruses, alphaviruses or orthonairoviruses without a major impact on livestock health could also be effective (340–343). As an example, Serbia started using calves as sentinels for WNV as a high seroprevalence in horses limits their use in surveillance (344). Many livestock samples are collected (**chapter 6**) in the Netherlands and thus offer potential for surveillance and potentially even repeated testing. Unfortunately, we were not able to test these species in this thesis, partially due to the aforementioned hesitancy for testing notifiable diseases combined with the need for owner approval. Even for non-notifiable viruses, hesitancy was present due to fear of media attention and subsequent losses in sales.

Wild birds are known reservoir hosts for most of the MBVs described in this thesis. Surveillance of live and dead wild birds has been extensively described in literature (76,345). In **chapter 3** SINV seropositivity in wild birds around a SINV positive bird and seropositive horses was 10.9%. A proportion of these birds was considered to be Dutch resident birds and thus, together with the seropositive horses, provides strong evidence for local transmission. A major advantage of research or surveillance of viruses like WNV, USUV and SINV in wild birds is the possibility for virus detection and thus phylogenetic analyses if a sequence can be obtained. This information can be analysed to study geographical spread and evolution of viruses (56,346), as was also done for the partial sequence in **chapter 3**. While virus detection in other species is possible, it is less likely to be successful. The Netherlands has an existing wild bird ringing infrastructure that is very useful for MBV surveillance (58,147). Disadvantages

of the use of wild birds are the limited option to resample animals, and the unknown origin or migration patterns of some birds.

Besides the studied species in this thesis, livestock and other animal species might be suitable for MBV surveillance. Multiple studies have been published on the use of various zoo animals, rodents and other wildlife species such as ungulates (92,93,100,194,347–349). In the Netherlands, studies in roe deer (*Capreolus capreolus*) have been performed but were focused specifically on TBEV (136,137,350). Use of zoo animals, rodents and other wildlife species for MBV surveillance in the Netherlands thus requires further study.

8.3 Serological methods & interpretation

Under optimal conditions, MBV infections can be confirmed through PCR, detection of immunoglobulin M (IgM) and/or a significant rise in (neutralizing) antibodies in combination with clinical signs (5,130,351). However, for non-recent infections, this confirmation is often not feasible. Interpretation of serological studies of arboviruses is hampered by the use (and lack of standardisation) of different assays, co-circulation of related viruses and cross-reaction of antibodies. Additionally, very limited knowledge is available about antibody kinetics over time, especially in animals. Different assays for serological diagnosis of arbovirus infections are used both within and across studies (352). Interpretation of the results from serosurveys using different assays is thus challenging, as is also shown in this thesis.

Generally, sera are first screened using an antibody binding assay such as an ELISA, Microsphere immunoassay (MIA) or Protein Microarray (PMA) (117). These assays can use different antigens, which for orthoflaviviruses are often the envelope (E) protein (as used in **chapters 2** and **4**) or non-structural 1 (NS1) protein (as used in **chapter 5**). The choice of assay format and antigen highly influences sensitivity and specificity of the assays (130,353). The E protein is involved in membrane fusion and is a major target for neutralizing antibodies (117). Amino acid homology of (specific domains of) the E protein is high among orthoflaviviruses, resulting in a cross-reactive (neutralizing) response (354). NS1 is required for virus replication but is also a highly immunogenic and important for the development of (non-neutralizing) protective antibodies (117,214). The use of (recombinant) NS1 is therefore often suggested as an alternative to the E protein for serological diagnosis to enable differentiation between cross-reactive orthoflaviviruses (214,214).

Positive screening samples can then be confirmed using a receptor-binding blocking assay such as HI, or a neutralization assay such as a Virus Neutralization Test (VNT), as gold standard. As cross-reactions may still occur in confirmatory assays, parallel tests

are ran for viruses within the serogroup that co-circulate in the area of interest. Diagnostic guidelines and algorithms from literature and the World Health Organization (WHO) and World Organisation for Animal Health (WOAH) recommend using a four-fold or greater difference between two titres to confirm infection (30,355,356). Interpretation by using a four-fold titre difference is based on a classification scheme for *Togaviridae* (genus *Alphavirus*) from the 80's (357). Forty-nine flaviviruses were grouped in antigenic complexes based on this classification scheme following experimental infections in mice (127). Both the probability and magnitude of cross-reaction, as well as waning of antibodies affect the development and persistence of (cross-reactive) neutralizing titres. The probability and magnitude of cross-reactivity may differ between to viruses, as we describe for USUV and WNV in **chapter 6**. Neutralizing titres are further complicated by original antigenic sin (OAS) and waning of antibodies. The phenomenon OAS means that if an animal gets infected with, for example, WNV after it already had been infected with USUV in the past, the neutralizing antibody response for USUV can be much stronger compared to that of the infecting virus (WNV). For many virus combinations, very little knowledge exists on the probability and extent of cross-reaction and OAS (352). Antibody persistence is also very rarely studied and may differ between viruses and species, as discussed in **chapter 5**. Studying this phenomenon can help us estimate the time past infection, and possibility for animals to become seronegative after a certain period. This is important in the view of using animals as sentinels as shown in **chapter 5**. In **chapter 6** I show that using statistical methods to disentangle serological outcomes may aid in understanding dynamics of cross-reactivity. These types of models provide promising approaches as they have been used for multiple serogroups, species and research questions of MBVs as well as viruses like influenza and SARS-CoV-2 (182,183,358).

The lack of representative confirmed positive and negative serum samples of multiple animal species further hampers analyses of sensitivity and specificity, which are needed for accurate seroprevalence calculations. Ideally, a historical group of sera from the same species and region is used as a negative control. Experimental infections or PCR/IgM confirmed cases are often used as positive controls. Unfortunately, such samples are often not available for the species of study. Even though species-independent assays such as specific ELISA formats (as in **chapter 2** and **4**) and VNT are used, it is known antibody development differs per species and thus may affect the outcomes and assay interpretation. It could be interesting to study sequential infections with multiple viruses in experimental settings, or acquire samples from field setting assess the cross-reactive responses in different animal species.

The world is rapidly changing, and changes in climate, land use, travel and trade will most likely result in introduction and spread of new emerging zoonotic MBVs. Introduction of new viruses will further complicate serological interpretation as co-circulation and related cross-reactions will become common. Therefore, we need (1) accurate knowledge on age, disease, vaccination status and origin of animals sampled within surveillance systems, (2) development of sensitive multitarget and serological tests that are able to capture immune responses to a broader range of viruses, (3) development of models that allow us to better interpret serological findings in a co-circulation context. Diagnostic panels should not be restrictive to known circulating viruses (e.g. WNV, USUV) but be able to detect viruses with low or unknown circulation (e.g. SINV, RVFV, JEV, Louping Ill virus, Bagaza virus).

8.4 Setting up One Health MBV research and surveillance in practice

Prioritization and planning surveillance activities for zoonotic MBVs requires effective communication between stakeholders to divide roles and responsibilities. The Dutch zoonoses structure involves many stakeholders from the public and animal health domains, each with its own jurisdiction and mandates (**chapter 6**). For some MBVs such as RVFV, there are legal obligations for surveillance and response practices which are already allocated at specific institutes. For other viruses, the surveillance objective should be agreed upon between relevant stakeholders (359), as this clarifies priorities and facilitates collaboration and data sharing. Decisions made for prioritization and plans of action with regards to surveillance and response sometimes lack transparency. Clear communication of these decisions enhance trust and understanding in follow-up procedures and allow for better collaboration (360). It further clarifies the responsibilities and goals for each stakeholder. While for MBV research a common objective among research institutes is not required, regular communication regarding aims and results with relevant stakeholders is recommended to ensure all parties remain informed and up-to-date.

Based on the findings in this thesis, the majority of Dutch horses and dogs are currently still susceptible for orthoflaviviruses which provides opportunities for surveillance for as well as detection of clinical cases. Detection of clinical cases requires awareness of owners and veterinarians, combined with accessible (low cost) diagnostic opportunities and cost compensation. Unclarity of response by the NVWA and procedures can be solved by clear response plans and communication about these plans. In high-risk regions, the use of sentinel backyard and petting zoo chickens may be very useful to indicate public health risk, because these chickens reside in or near urban areas with high human densities. Wild boars are ideal candidates for continuous surveillance as samples are already collected and stored but their geographic range may be limiting. Similar sampling streams are available for livestock, but further

research is needed to investigate the potential of commercial poultry and other livestock species for sustainable MBV surveillance. Use of livestock samples requires removal of the hesitancy for sharing samples that will be tested for notifiable or emerging pathogens. Also, the effect of different housing systems on MBV exposure needs to be evaluated. This is not only interesting for surveillance purposes but also may result in higher or lower risk for future agricultural systems.

Many promising sample streams and existing collaborations do already exist, which can be built upon when designing a sustainable research and surveillance plan for MBVs. Responsibilities of laboratory diagnostics, case detection, communication and control need to be divided between stakeholders and clearly communicated. Awareness of the general public, veterinary and human health professionals relies on robust communication channels that reach the target audience. For veterinarians such a communication channel does currently not exist. The government should anticipate and enhance preparedness, as outbreaks of MBVs are likely to become more frequent in the future. Dutch One Health stakeholders should thus, collectively, prepare response plans for outbreaks that are yet to come. This also includes land use planning and climate adaptation decisions, as they have an effect on MBV outbreak risk as shown in **chapter 7**. Furthermore, biobanking of relevant samples with sufficient accompanying metadata provides the option for rapid response surveillance, or serve as reference samples of specific populations as indicated in **chapter 6**.

8.5 Ethical considerations of surveillance and control options

Research and surveillance of zoonotic MBVs, as described in this thesis, often makes use of animal samples. In most cases, blood or serum samples of animals are sufficient to provide information on the circulation and spread of MBVs. As shown in Figure 1 and described by the Dutch Emerging Zoonoses Information and Priority system (EZIPs) list, prevention of impacts of outbreaks on public health or the economy are often the main goals of surveillance. However, public health and economic interests may easily conflict with animal welfare or ecosystem health in zoonotic disease surveillance or control (361). One clear example is the culling of healthy animals during zoonotic disease outbreaks. In case of significant human health risks, the use of animals in surveillance can be justified (361,362). But what defines ‘significant human health risks’? And what about viruses with a high impact on public health but a negligible risk for animal health?

We could question whether (invasive) sampling of animals is justified if we are looking for viruses with a limited impact on human health or the economy. A simple solution could be the reuse of already collected samples, non-invasive samples, or use of

environmental samples as described in **chapter 4** and **6**. For some viruses we might be able to directly tackle the risk for public health by using available vaccines in humans, animals, or both. An example is TBEV, for which commercial vaccines are available in Europe. Currently, TBEV vaccination is not actively promoted nor frequently applied in humans in the Netherlands, because of the low infection risk for humans (142). Even potential high risk groups such as bird ringers have a very low vaccination rate of about 5% (8/157) (147). This could be increased by actively promoting vaccination in risk groups, thereby lowering disease risk for relevant groups. Additionally, the use of existing samples of wildlife and (small) ruminants, or even non-invasive samples like cheese and milk could be used in surveillance as a relatively simple and potentially cost-effective approach.

Selection of viruses to survey (and potentially research funding) is often done based on ranking their impact, which in some cases is incorporated in legislation. An interesting example of an emerging threat is Japanese Encephalitis Virus (JEV). JEV is placed 3rd on the Dutch EZIPs list, but has the lowest ranking (E) in the European Animal Health Law (AHL) (113,363). In contrast to the EZIPs classification, the AHL does take into account animal health and welfare impacts. The high EZIPs classification of JEV is mainly due to the potential economic damage and high human mortality. For the AHL, the expert panel did not list JEV as highly transmissible. No consensus could be reached about whether the disease has a significant environmental or animal welfare impact, or whether it results in high morbidity and significant mortality rates in animals. The AHL listing report quotes from the Australian response strategy (364): "*due to the time taken to establish a diagnosis, continuing virus transmission by insects, the wide range of hosts and the lack of clinical signs in most infected animals, control by slaughtering exposed animals is inappropriate*." This raises the question for a virus that is that high on the EZIPs list, but slaughtering exposed animals is not an option, how we would future control outbreaks?

Control of future MBV outbreaks becomes particularly intriguing when considering the contrast between JEV and RVFV. The placement of RVFV as 11th on the EZIPs is mainly because of lower human mortality compared to JEV. In contrast, it is an A listed disease by the AHL and control of outbreaks by culling of ruminants is mandatory in the EU. Currently, no JEV nor RVFV vaccines registered for animals in EU. In my opinion, preparedness for JEV and RVFV is essential as outbreaks would have a large impact, whether it is on human health, animal health, their welfare or the environment. Especially since there are no, or only very drastic control options, early-detection of these viruses is crucial. The results of **chapter 7** underscore the need for preparedness as the RVFV outbreak potential and duration of the transmission season will likely increase in the coming decades. Preparedness would include the development of surveillance and response plans, and include ethical considerations to weigh the

importance of public health, animal health and the economy, combined with efforts to develop and license effective vaccines.

Early detection of MBV circulation is useful when appropriate prevention and control measures can be taken to prevent new cases in animals or humans (Figure 1). Control options for MBV outbreaks are often limited, which leads to the question what are actionable responses to early detection or outbreaks of MBVs. Current mosquito control strategies (such as pesticide application) often involve risks for the natural environment. With regards to animals, control of outbreaks for viruses with a domestic animal reservoir might include culling and movement or trade restrictions. For humans, prevention of mosquito bites and removal of breeding sites are currently the main pillars in communication strategies from the Dutch government to prevent infections. Additionally, according to European legislation, blood donations from an outbreak region will be molecularly tested to prevent transmission via blood products (313). Nevertheless, the limited effective control options highlight the need for resilient landscape configuration and development of safe and effective vaccines. As there are substantial differences in tissue tropism, pathogenicity and outcome of disease between viral species, these need to be taken into account when developing vaccines (60). Until safe and effective vaccines become available, publicly available action plans are needed to enhance transparency and trust between all stakeholders. This preparedness is required to detect cases early and already develop tools for vaccines and decide on vaccination strategies (365).

Zoonotic diseases are part of life and a certain level of risk is thus unavoidable, although MBV outbreaks may have a significant impact on human and animal health, the environment and economy. Even though these outbreaks can have profound impacts, they must be understood within the broader context of global health challenges. Effective policy decisions require not only scientific understanding but also informed public engagement, acknowledging the complexity of these risks. Policy decisions on prioritization thus requires a broad and informative public dialogue (361).

8.6 Results of this thesis in relation to the OHPACT consortium

The research consortium (OHPACT) aimed to gain systemic understanding of mosquito-borne diseases, and of how their emergence and transmission is influenced by major environmental and social changes. This thesis has provided insight in the baseline seroprevalence of WNV, USUV and SINV in horses and dogs (**chapter 2** and **3**) and undetected circulation of WNV and USUV (**chapter 4** and **5**). These studies have shown that USUV, WNV and SINV have been circulating despite the fact that no human nor equine cases were (officially) reported. In accordance with our results in horses and dogs, a study in bird ringers indicated a low level of exposure of WNV and USUV in the Netherlands (147). This study also indicated the complexity of interpreting

serological results in these low-prevalence settings, as I have also shown in this thesis. Tools that might aid in improving arbovirus surveillance such as alternative sample types or fieldable assays have been investigated and/or developed within the consortium and could potentially be applied in MBV research and surveillance in animals (71,366). Next to these options for alternative sample types and diagnostic methods, we have identified promising sample streams that could be used in MBV surveillance (**chapter 6**).

Design of MBV research and surveillance programmes largely depend on their transmission cycle and thus their vectors. Therefore, studies on vectorial capacity are of great importance (367). Studies from OHPACT show the complexity of mosquito population dynamics and behaviour, and thus their role in arbovirus transmission and overwintering (35,169,219,368,369). Even more complexity comes into play when we take into account the role of birds, humans (or other host species), co-infections with other diseases and environmental factors (370–372). Constant increase in knowledge of the transmission cycle is gathered and allows for better understanding of the current transmission dynamics (370). This knowledge can be used to develop more refined models to understand or anticipate MBV transmission (370).

Even though understanding current transmission dynamics is already complicated, researchers within OHPACT have attempted to predict what might happen in the future. One Health based future scenarios were developed for the Netherlands (112). Based on these scenarios, land use maps were created that allow us to model mosquito and host populations on a more detailed level (303). Predictions of *Cx. pipiens/torrentium* populations (291) were subsequently created using the scenarios and land use maps by Dellar et al. (303). The scenarios, land use maps and mosquito predictions were all used in **chapter 7** of this thesis. Integration of these methods allowed us to detect differences between future scenarios (SSP1 and SSP5) that are likely due to differences in drivers such as the rise in temperature and land use changes combined with policy decisions that lead to changes in the number and distribution of livestock species.

8.7 Conclusions and further research

One Health surveillance for zoonotic arboviruses has to be based on a commonly agreed goal. For this goal, the importance of animal and environmental health should not be overruled by public health or economic interests. In ideal situations, we would be able to detect viral circulation early (or even predict) before clinical cases in human or animals occur (Figure 1). However, such practices are often costly and labor-intensive, raising the question of whether the effort is justified in achieving the intended goals. Conducting cost-efficiency analyses is crucial to designing sustainable surveillance systems that, ideally, fit a multi-virus purpose.

Animals can be very useful in MBV surveillance, but not all species are equally suitable in the Netherlands. Lowering the barriers for notification of suspected cases, combined with the development and availability of diagnostic tests will support early-detection by syndromic surveillance. Active surveillance can be performed in high-risk regions by using sentinel chickens, mosquitoes and wild birds. Additionally, existing systems and sample streams provide extra information on the introduction, local spread and/or endemicity of zoonotic arboviruses. We should make use of the extensive veterinary and food safety systems that are already in place. Further research is required to investigate the potential use of specific livestock species, zoo animals, wild rodents and ungulates as well as other sample types that are less invasive.

Fundamental research is needed to generate knowledge on (species specific) immune responses, cross-reaction and antibody waning in animals. This information can be used in statistical models to interpret serological surveillance results. Furthermore, models to predict (the impact of) MBV outbreaks require local estimates for vector competence, host-feeding behaviour and heterogeneity in MBV transmission cycles. A clear communication and distribution of responsibilities between academic, governmental and commercial partners can help to acquire this knowledge and jointly design an appropriate sustainable zoonotic arbovirus surveillance system for the Netherlands.

8.8 Outlook

In a densely populated country as the Netherlands, it is essential to acknowledge we have a shared responsibility to safeguard environmental, animal and human health and welfare. Even though zoonoses may be a part of the natural world around us, drivers of emergence of zoonotic MBVs are often related to human behaviour. It is important to avoid creating a perfect habitat for emerging MBVs in a country with a high livestock density, many wetland areas and increasing human-wildlife-livestock interface. Not only will researchers and other stakeholders have to focus on developing disease prevention measures like vaccines and biosafety measures. The focus should be on configuring our landscape to build resilient ecosystems that can mitigate the impact of MBV outbreaks. This includes dealing with underlying problems such as climate change and biodiversity loss. Only in this way, we can create a sustainable and healthy country for all ecosystems.

"*My only wish, in presenting this communication, is that note be taken of my observations, and that the truth of my suspicions and conceptions be left to the decisive evidence furnished by direct experimentation. This does not signify, however, that I am at all desirous of evading the discussion of the views which I have just enunciated; on the contrary, it will be with great pleasure that I will listen to any remarks or objections which my distinguished colleagues may deem proper to make*" - Dr Charles Finlay, Havana, August 11th 1881

References

1. Finlay C. The mosquito hypothetically considered as an agent in the transmission of yellow fever poison. The New Orleans Medical and Surgical Journal. 1881;9:601–16.

2. Jesse William Lazear. Science. 1900 Dec 14;12(311):932–3.

3. One Health PACT - Predicting Arboviruses Climate Tipping Points [Internet]. 2020. Available from: https://www.onehealthpact.org/about/

4. CDC. Center for Disease Control and Prevention. [cited 2024 Mar 8]. Arbovirus Catalog. Available from: https://wwwn.cdc.gov/ArboCAT/History.aspx

5. WOAH. Terrestrial Animal Health Code [Internet]. 31st ed. Paris: World Organisation for Animal Health; 2023. Available from: https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/

6. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. 2008;451(7181):990–3.

7. Castro MC, Wilson ME, Bloom DE. Disease and economic burdens of dengue. The Lancet Infectious Diseases. 2017 Mar;17(3):e70–8.

8. WHO. Dengue and Severe Dengue [Internet]. 2023 [cited 2024 May 4]. Available from: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

9. Stanaway JD, Shepard DS, Undurraga EA, Halasa YA, Coffeng LE, Brady OJ, et al. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. The Lancet Infectious Diseases. 2016 Jun;16(6):712–23.

10. PAHO. Dengue Epidemiological Situation in the Region of the Americas - Epidemiological Week 20, 2024 [Internet]. Pan American Health Organization; 2024. Report No.: 20. Available from: https://www.paho.org/en/documents/situation-report-no-20-dengue-epidemiological-situation-region-americas-epidemiological

11. Hubálek Z, Rudolf I, Nowotny N. Arboviruses Pathogenic for Domestic and Wild Animals. In: Advances in Virus Research. Elsevier; 2014. p. 201–75.

12. Lefkowitz EJ, Dempsey DM, Hendrickson RC, Orton RJ, Siddell SG, Smith DB. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). Nucleic Acids Research. 2018 Jan 4;46(D1):D708–17.

13. Liang G, Gao X, Gould EA. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. Emerging Microbes & Infections. 2015 Jan 1;4(1):1–5.

14. Rosselló J, Santana-Gallego M, Awan W. Infectious disease risk and international tourism demand. Health Policy and Planning. 2017 May 1;32(4):538–48.

15. IFRC, UNDP. A Socio-economic Impact Assessment of the Zika Virus in Latin America and the Caribbean: with a focus on Brazil, Colombia and Suriname [Internet]. United Nations Development Programme (UNDP) and International Federation of the Red Cross and Red Crescent Societies (IFRC); 2017. Available from: https://www.undp.org/publications/socio-economic-impact-assessment-zika-virus-latin-america-and-caribbean

16. de Vos CJ, Hennen WHGJ, van Roermund HJW, Dhollander S, Fischer EAJ, de Koeijer AA. Assessing the introduction risk of vector-borne animal diseases for the Netherlands using MINTRISK: A Model for INTegrated RISK assessment. Dórea FC, editor. PLoS ONE. 2021 Nov 2;16(11):e0259466.

17. Bron GM, Strimbu K, Cecilia H, Lerch A, Moore SM, Tran Q, et al. Over 100 Years of Rift Valley Fever: A Patchwork of Data on Pathogen Spread and Spillover. Pathogens. 2021 Jun 5;10(6):708.

18. Nin Pratt A, Bonnet P, Jabbar MA, Ehui S, de Haan C. Benefits and costs of compliance of sanitary regulations in livestock markets: the case of Rift Valley fever in the Somali Region of Ethiopia. Nairobi: ILRI (International Livestock Research Institute); 2005. 70 p.

19. Brühl CA, Després L, Frör O, Patil CD, Poulin B, Tetreau G, et al. Environmental and socioeconomic effects of mosquito control in Europe using the biocide *Bacillus thuringiensis* subsp. *israelensis* (Bti). Science of The Total Environment. 2020 Jul;724:137800.

20. Kilpatrick AM, Wheeler SS. Impact of West Nile Virus on Bird Populations: Limited Lasting Effects, Evidence for Recovery, and Gaps in Our Understanding

of Impacts on Ecosystems. Reisen W, editor. Journal of Medical Entomology. 2019 Oct 28;56(6):1491–7.

21. Weaver SC, Barrett ADT. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Microbiol. 2004 Oct;2(10):789–801.

22. Turell MJ. Members of the *Culex pipiens* Complex as Vectors of Viruses. Journal of the American Mosquito Control Association. 2012 Dec;28(4s):123–6.

23. Kraemer MUG, Reiner RC, Brady OJ, Messina JP, Gilbert M, Pigott DM, et al. Past and future spread of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. Nat Microbiol. 2019 Mar 4;4(5):854–63.

24. ECDC, EFSA. European Center for Disease Prevention and Control (ECDC). 2023 [cited 2024 Jul 13]. Mosquito maps. Available from: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps

25. Ramírez AL, Van Den Hurk AF, Meyer DB, Ritchie SA. Searching for the proverbial needle in a haystack: advances in mosquito-borne arbovirus surveillance. Parasites Vectors. 2018 Dec;11(1):320.

26. Mansfield KL, Hernández-Triana LM, Banyard AC, Fooks AR, Johnson N. Japanese encephalitis virus infection, diagnosis and control in domestic animals. Veterinary Microbiology. 2017 Mar;201:85–92.

27. Frank C, Schmidt-Chanasit J, Ziegler U, Lachmann R, Preußel K, Offergeld R. West Nile Virus in Germany: An Emerging Infection and Its Relevance for Transfusion Safety. Transfus Med Hemother. 2022 Aug;49(4):192–204.

28. Jimenez A, Shaz BH, Bloch EM. Zika Virus and the Blood Supply: What Do We Know? Transfus Med Rev. 2017 Jan;31(1):1–10.

29. ECDC. West Nile virus infection. Annual Epidemiological Report for 2018. [Internet]. Stockholm: European Centre for Disease Prevention and Control; 2019. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/west-nile-fever-annual-epidemiological-report-2018.pdf

30. WOAH. West Nile Fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 8th ed. Office International des Epizooties; 2018. p. Chapter 2.1.24.

31. Chala B, Hamde F. Emerging and Re-emerging Vector-Borne Infectious Diseases and the Challenges for Control: A Review. Front Public Health. 2021 Oct 5;9:715759.

32. Swei A, Couper LI, Coffey LL, Kapan D, Bennett S. Patterns, Drivers, and Challenges of Vector-Borne Disease Emergence. Vector-Borne and Zoonotic Diseases. 2020 Mar 1;20(3):159–70.

33. Pearce-Higgins JW, Green RE. Birds and climate change: impacts and conservation responses. Cambridge (GB): Cambridge university press; 2014. (Ecology, biodiversity and conservation).

34. Franklinos LHV, Jones KE, Redding DW, Abubakar I. The effect of global change on mosquito-borne disease. The Lancet Infectious Diseases. 2019 Sep;19(9):e302–12.

35. Krol L, Blom R, Dellar M, Van Der Beek JG, Stroo ACJ, Van Bodegom PM, et al. Interactive effects of climate, land use and soil type on *Culex pipiens/torrentium* abundance. One Health. 2023 Dec;17:100589.

36. Pfeffer M, Dobler G. Emergence of zoonotic arboviruses by animal trade and migration. Parasites Vectors. 2010;3(1):35.

37. Ibáñez-Justicia A, Smitz N, Den Hartog W, Van De Vossenberg B, De Wolf K, Deblauwe I, et al. Detection of Exotic Mosquito Species (Diptera: Culicidae) at International Airports in Europe. IJERPH. 2020 May 15;17(10):3450.

38. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz Soldan V, Kochel TJ, Kitron U, et al. The Role of Human Movement in the Transmission of Vector-Borne Pathogens. Kittayapong P, editor. PLoS Negl Trop Dis. 2009 Jul 21;3(7):e481.

39. Benedict MQ, Levine RS, Hawley WA, Lounibos LP. Spread of The Tiger: Global Risk of Invasion by The Mosquito *Aedes albopictus*. Vector-Borne and Zoonotic Diseases. 2007 Mar;7(1):76–85.

40. Ibáñez-Justicia A, Alcaraz-Hernández JD, Van Lammeren R, Koenraadt CJM, Bergsma A, Delucchi L, et al. Habitat suitability modelling to assess the introductions of *Aedes albopictus* (Diptera: Culicidae) in the Netherlands. Parasites Vectors. 2020 Dec;13(1):217.

41. Gottdenker NL, Streicker DG, Faust CL, Carroll CR. Anthropogenic Land Use Change and Infectious Diseases: A Review of the Evidence. EcoHealth. 2014 Dec;11(4):619–32.

42. Norris DE. Mosquito-borne Diseases as a Consequence of Land Use Change. EcoHealth. 2004 Mar 1;1(1):19–24.

43. Walsh MG, Webb C, Brookes V. An evaluation of the landscape structure and La Niña climatic anomalies associated with Japanese encephalitis virus outbreaks reported in Australian piggeries in 2022. One Health. 2023 Jun;16:100566.

44. Walsh MG, Pattanaik A, Vyas N, Saxena D, Webb C, Sawleshwarkar S, et al. High-risk landscapes of Japanese encephalitis virus outbreaks in India converge on wetlands, rain-fed agriculture, wild Ardeidae, and domestic pigs and chickens. International Journal of Epidemiology. 2022 Oct 13;51(5):1408–18.

45. Suk JE, Semenza JC. Future Infectious Disease Threats to Europe. Am J Public Health. 2011 Nov;101(11):2068–79.

46. European Environment Agency. European climate risk assessment: executive summary. [Internet]. LU: Publications Office; 2024 [cited 2024 Apr 5]. Available from: https://data.europa.eu/doi/10.2800/204249

47. European Union. Climate change: what the EU is doing [Internet]. 2024 [cited 2024 Apr 5]. Available from: https://www.consilium.europa.eu/en/policies/climate-change/#:~:text=Under%20the%20European%20climate%20law,EU%20climate%20neutral%20by%202050.&text=Why%20is%20the%20green%20transition%20necessary%3F

48. European Commission. Forging a climate-resilient Europe - the new EU Strategy on Adaptation to Climate Change [Internet]. 2021 [cited 2024 May 4]. Available from: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=COM:2021:82:FIN

49. Rocklöv J, Dubrow R. Climate change: an enduring challenge for vector-borne disease prevention and control. Nat Immunol. 2020 May 1;21(5):479–83.

50. ECDC, EFSA. The importance of vector abundance and seasonality: results from an expert consultation. [Internet]. Vol. European Center for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA). LU: Publications Office; 2018 [cited 2024 Apr 8]. Available from: https://data.europa.eu/doi/10.2900/37171

51. Koch RT, Erazo D, Folly AJ, Johnson N, Dellicour S, Grubaugh ND, et al. Genomic epidemiology of West Nile virus in Europe. One Health. 2024 Jun;18:100664.

52. Malkinson M, Banet C. The Role of Birds in the Ecology of West Nile Virus in Europe and Africa. In: Mackenzie JS, Barrett ADT, Deubel V, editors. Japanese Encephalitis and West Nile Viruses. Berlin, Heidelberg: Springer Berlin Heidelberg; 2002. p. 309–22. (Compans RW, Cooper MD, Ito Y, Koprowski H, Melchers F, Oldstone MBA, et al., editors. Current Topics in Microbiology and Immunology; vol. 267).

53. Clé M, Beck C, Salinas S, Lecollinet S, Gutierrez S, Van de Perre P, et al. Usutu virus: A new threat? Epidemiol Infect. 2019;147:e232.

54. Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of *Usutu virus*, an African Mosquito-Borne *Flavivirus* of the Japanese Encephalitis Virus Group, Central Europe. Emerg Infect Dis. 2002 Jul;8(7):652–6.

55. Rijks J, Kik M, Slaterus R, Foppen R, Stroo A, IJzer J, et al. Widespread Usutu virus outbreak in birds in the Netherlands, 2016. Eurosurveillance. 2016 Nov 10;21(45).

56. Oude Munnink BB, Münger E, Nieuwenhuijse DF, Kohl R, van der Linden A, Schapendonk CME, et al. Genomic monitoring to understand the emergence and spread of Usutu virus in the Netherlands, 2016–2018. Sci Rep. 2020 Feb 18;10(1):2798.

57. Vlaskamp DR, Thijsen SF, Reimerink J, Hilkens P, Bouvy WH, Bantjes SE, et al. First autochthonous human West Nile virus infections in the Netherlands, July to August 2020. Eurosurveillance. 2020 Nov 19;25(46).

58. Sikkema RS, Schrama M, van den Berg T, Morren J, Munger E, Krol L, et al. Detection of West Nile virus in a common whitethroat (*Curruca communis*) and *Culex* mosquitoes in the Netherlands, 2020. Eurosurveillance. 2020 Oct 8;25(40).

59. Ling J, Smura T, Lundström JO, Pettersson JHO, Sironen T, Vapalahti O, et al. Introduction and Dispersal of Sindbis Virus from Central Africa to Europe. Heise MT, editor. J Virol. 2019 Aug 15;93(16):e00620-19.

60. van Bree JWM, Visser I, Duyvestyn JM, Aguilar-Bretones M, Marshall EM, van Hemert MJ, et al. Novel approaches for the rapid development of rationally designed arbovirus vaccines. One Health. 2023 Jun;16:100565.

61. Potter A, Jardine A, Morrissey A, Lindsay MDA. Evaluation of a Health Communication Campaign to Improve Mosquito Awareness and Prevention Practices in Western Australia. Front Public Health. 2019 Mar 19;7:54.

62. ECDC, EFSA. Surveillance, prevention and control of West Nile virus and Usutu virus infections in the EU/EEA [Internet]. Stockholm: European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA); 2023 Sep [cited 2024 Mar 8]. Available from: https://data.europa.eu/doi/10.2903/sp.efsa.2023.EN-8242

63. EFSA. Rift Valley Fever – assessment of effectiveness of surveillance and control measures in the EU. EFSA Journal. 2020 Nov;18(11).

64. FAO, WHO. Rift Valley fever outbreaks forecasting models. [Internet]. Rome, Italy: World Health Organization (WHO) and Food and Agriculture Organization (FAO); 2009. Report No.: WHO/HSE/GAR/BDP/2009.2. Available from: https://www.who.int/publications/i/item/rift-valley-fever-outbreaks-forecasting-models

65. Wimberly MC, Davis JK, Hildreth MB, Clayton JL. Integrated Forecasts Based on Public Health Surveillance and Meteorological Data Predict West Nile Virus in a High-Risk Region of North America. Environ Health Perspect. 2022 Aug;130(8):087006.

66. Braks M, Van Der Giessen J, Kretzschmar M, Van Pelt W, Scholte EJ, Reusken C, et al. Towards an integrated approach in surveillance of vector-borne diseases in Europe. Parasites Vectors. 2011 Dec;4(1):192.

67. Yu X, Cheng G. Adaptive Evolution as a Driving Force of the Emergence and Re-Emergence of Mosquito-Borne Viral Diseases. Viruses. 2022 Feb 21;14(2):435.

68. Rathore APS, St. John AL. Cross-Reactive Immunity Among Flaviviruses. Front Immunol. 2020 Feb 26;11:334.

69. Salman MD. Surveillance tools and strategies for animal diseases in a shifting climate context. Anim Health Res Rev. 2013 Dec;14(2):147–50.

70. Ahmed W, Liu Y, Smith W, Ingall W, Belby M, Bivins A, et al. Harnessing Wastewater Surveillance to Detect Livestock-Linked Viruses [Internet]. 2023 [cited 2024 Jun 14]. Available from: https://www.preprints.org/manuscript/202309.1616/v1

71. Atama NC, Chestakova IV, De Bruin E, Van Den Berg TJ, Munger E, Reusken C, et al. Evaluation of the use of alternative sample types for mosquito-borne flavivirus surveillance: Using Usutu virus as a model. One Health. 2022 Dec;15:100456.

72. Sakata MK, Sato M, Sato MO, Watanabe T, Mitsuishi H, Hikitsuchi T, et al. Detection and persistence of environmental DNA (eDNA) of the different developmental stages of a vector mosquito, *Culex pipiens pallens*. Doi H, editor. PLoS ONE. 2022 Aug 10;17(8):e0272653.

73. Calzolari M, Gaibani P, Bellini R, Defilippo F, Pierro A, Albieri A, et al. Mosquito, Bird and Human Surveillance of West Nile and Usutu Viruses in Emilia-Romagna Region (Italy) in 2010. Coffey LL, editor. PLoS ONE. 2012 May 30;7(5):e38058.

74. Constant O, Gil P, Barthelemy J, Bolloré K, Foulongne V, Desmetz C, et al. One Health surveillance of West Nile and Usutu viruses: a repeated cross-sectional study exploring seroprevalence and endemicity in Southern France, 2016 to 2020. Eurosurveillance. 2022 Jun 23;27(25).

75. EFSA AHAW panel, More S, Bøtner A, Butterworth A, Calistri P, Depner K, et al. Ad hoc method for the assessment on listing and categorisation of animal diseases within the framework of the Animal Health Law. EFSA Journal [Internet]. 2017 Jul [cited 2024 Jun 14];15(7). Available from: https://data.europa.eu/doi/10.2903/j.efsa.2017.4783

76. Komar N. West Nile Virus Surveillance using Sentinel Birds. Annals of the New York Academy of Sciences. 2001 Dec;951(1):58–73.

77. Leblond A, Hendrikx P, Sabatier P. West Nile Virus Outbreak Detection Using Syndromic Monitoring in Horses. Vector-Borne and Zoonotic Diseases. 2007 Sep;7(3):403–10.

78. McLean RG, Ubico SR, Docherty DE, Hansen WR, Sileo L, McNamara TS. West Nile Virus Transmission and Ecology in Birds. Annals of the New York Academy of Sciences. 2001 Dec;951(1):54–7.

79. Plowright RK, Parrish CR, McCallum H, Hudson PJ, Ko AI, Graham AL, et al. Pathways to zoonotic spillover. Nat Rev Microbiol. 2017 Aug;15(8):502–10.

80. Beck C, Leparc Goffart I, Franke F, Gonzalez G, Dumarest M, Lowenski S, et al. Contrasted Epidemiological Patterns of West Nile Virus Lineages 1 and 2 Infections in France from 2015 to 2019. Pathogens. 2020 Oct 30;9(11):908.

81. Thrusfield M. Veterinary epidemiology. 4th ed. Hoboken: Wiley Blackwell; 2018.

82. Burrell GA, Seibert FM, Mines USB of. Gases Found in Coal Mines [Internet]. U.S. Government Printing Office; 1916. (Miners' circular). Available from: https://books.google.nl/books?id=bit8KBOQCdUC

83. Stahl RG. Can mammalian and non-mammalian "sentinel species" data be used to evaluate the human health implications of environmental contaminants? Human and Ecological Risk Assessment: An International Journal. 1997 Jul;3(3):329–35.

84. Racloz V, Griot C, Stärk KDC. Sentinel surveillance systems with special focus on vector-borne diseases. Anim Health Res Rev. 2006 Jun;7(1–2):71–9.

85. Halliday JEB, Meredith AL, Knobel DL, Shaw DJ, Bronsvoort BM de C, Cleaveland S. A framework for evaluating animals as sentinels for infectious disease surveillance. J R Soc Interface. 2007 Oct 22;4(16):973–84.

86. Amdouni J, Monaco F, Portanti O, Sghaier S, Conte A, Hassine TB, et al. Detection of enzootic circulation of a new strain of West Nile virus lineage 1 in sentinel chickens in the north of Tunisia. Acta Tropica. 2020 Feb;202:105223.

87. Chaskopoulou A, Dovas CI, Chaintoutis SC, Kashefi J, Koehler P, Papanastassopoulou M. Detection and Early Warning of West Nile Virus Circulation in Central Macedonia, Greece, Using Sentinel Chickens and Mosquitoes. Vector-Borne and Zoonotic Diseases. 2013 Oct;13(10):723–32.

88. Kluh S, Madon MB, Nguyen DV, Barker CM, Reisen WK, Kwan JL. Sentinel Chicken Seroconversions Track Tangential Transmission of West Nile Virus to

Humans in the Greater Los Angeles Area of California. The American Journal of Tropical Medicine and Hygiene. 2010 Nov 5;83(5):1137–45.

89. McIntire S, Esposito M, Badger J. Sentinel Chickens and Their Role in Mosquito-Borne Virus Surveillance in Delaware. Delaware Journal of Public Health. 2021 Jan;7(1):72–4.

90. Morris CD, Baker WG, Stark L, Burgess J, Lewis AL. Comparison of chickens and pheasants as sentinels for eastern equine encephalitis and St. Louis encephalitis viruses in Florida. J Am Mosq Control Assoc. 1994 Dec;10(4):545–8.

91. Caballero-Gómez J, Cano-Terriza D, Lecollinet S, Carbonell MD, Martínez-Valverde R, Martínez-Nevado E, et al. Evidence of exposure to zoonotic flaviviruses in zoo mammals in Spain and their potential role as sentinel species. Veterinary Microbiology. 2020 Aug;247:108763.

92. Constant O, Bollore K, Clé M, Barthelemy J, Foulongne V, Chenet B, et al. Evidence of Exposure to USUV and WNV in Zoo Animals in France. Pathogens. 2020 Nov 30;9(12):1005.

93. Kvapil P, Račnik J, Kastelic M, Bártová E, Korva M, Jelovšek M, et al. A Sentinel Serological Study in Selected Zoo Animals to Assess Early Detection of West Nile and Usutu Virus Circulation in Slovenia. Viruses. 2021 Apr 6;13(4):626.

94. Currenti L, Tasca P, Díaz MDP, Contigiani M, Spinsanti L. Serological survey for Saint Louis encephalitis virus and West Nile virus in domestic mammals in Córdoba, Argentina: are our pets potential sentinels? Arch Virol. 2020 Sep;165(9):2079–82.

95. Durand B, Haskouri H, Lowenski S, Vachiery N, Beck C, Lecollinet S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: dog as an alternative WNV sentinel species? Epidemiol Infect. 2016 Jul;144(9):1857–64.

96. Laidoudi Y, Durand G, Watier-Grillot S, Dessimoulie AS, Labarde C, Normand T, et al. Evidence of Antibodies against the West Nile Virus and the Usutu Virus in Dogs and Horses from the Southeast of France. Casal J, editor. Transboundary and Emerging Diseases. 2023 Mar 24;2023:1–8.

97. Montagnaro S, Piantedosi D, Ciarcia R, Loponte R, Veneziano V, Fusco G, et al. Serological Evidence of Mosquito-Borne Flaviviruses Circulation in Hunting Dogs in Campania Region, Italy. Vector-Borne and Zoonotic Diseases. 2019 Feb;19(2):142–7.

98. Resnick MP, Grunenwald P, Blackmar D, Hailey C, Bueno R, Murray KO. Juvenile Dogs as Potential Sentinels for West Nile Virus Surveillance. Zoonoses and Public Health. 2008 Oct;55(8–10):443–7.

99. Cappelle J, Duong V, Pring L, Kong L, Yakovleff M, Prasetyo DB, et al. Intensive Circulation of Japanese Encephalitis Virus in Peri-urban Sentinel Pigs near Phnom Penh, Cambodia. Barrera R, editor. PLoS Negl Trop Dis. 2016 Dec 7;10(12):e0005149.

100. Escribano-Romero E, Lupulović D, Merino-Ramos T, Blázquez AB, Lazić G, Lazić S, et al. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. Veterinary Microbiology. 2015 Apr;176(3–4):365–9.

101. Mohammed MN, Yasmin AR, Ramanoon SZ, Noraniza MA, Ooi PT, Ain-Najwa MY, et al. Serological and molecular surveillance of West Nile virus in domesticated mammals of peninsular Malaysia. Front Vet Sci. 2023 Jun 29;10:1126199.

102. Gibbs SEJ, Hoffman DM, Stark LM, Marlenee NL, Blitvich BJ, Beaty BJ, et al. Persistence of Antibodies to West Nile Virus in Naturally Infected Rock Pigeons (*Columba livia*). Clin Vaccine Immunol. 2005 May;12(5):665–7.

103. Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K, et al. West Nile Virus and Usutu Virus Monitoring of Wild Birds in Germany. IJERPH. 2018 Jan 22;15(1):171.

104. Michel F, Sieg M, Fischer D, Keller M, Eiden M, Reuschel M, et al. Evidence for West Nile Virus and Usutu Virus Infections in Wild and Resident Birds in Germany, 2017 and 2018. Viruses. 2019 Jul 23;11(7):674.

105. Mullenix J, Hamm J, McLean RG, Kerschner J. The House Sparrow (*Passer domesticus*) as a Sentinel for St. Louis Encephalitis Virus. The American Journal of Tropical Medicine and Hygiene. 1983 Sep 1;32(5):1120–9.

106. Björnström A, Blomström AL, Singh MC, Hesson JC. Sindbis virus neutralising antibodies detected in Swedish horses. One Health. 2021 Jun;12:100242.

107. Gothe LMR, Ganzenberg S, Ziegler U, Obiegala A, Lohmann KL, Sieg M, et al. Horses as Sentinels for the Circulation of Flaviviruses in Eastern–Central Germany. Viruses. 2023 Apr 30;15(5):1108.

108. García-Bocanegra I, Arenas-Montes A, Jaén-Téllez JA, Napp S, Fernández-Morente M, Arenas A. Use of sentinel serosurveillance of mules and donkeys in the monitoring of West Nile virus infection. The Veterinary Journal. 2012 Nov;194(2):262–4.

109. Bournez L, Umhang G, Faure E, Boucher JM, Boué F, Jourdain E, et al. Exposure of Wild Ungulates to the Usutu and Tick-Borne Encephalitis Viruses in France in 2009–2014: Evidence of Undetected Flavivirus Circulation a Decade Ago. Viruses. 2019 Dec 19;12(1):10.

110. Santaella J, McLean R, Hall JS, Gill JS, Bowen RA, Hadow HH, et al. West Nile virus serosurveillance in Iowa white-tailed deer (1999-2003). Am J Trop Med Hyg. 2005 Dec;73(6):1038–42.

111. ANV. National Risk Assessment [Internet]. National Network of Safety and Security Analysts (ANV); 2019. Available from: https://english.nctv.nl/documents/publications/2019/09/18/dutch-national-risk-assessment

112. Dellar M, Geerling G, Kok K, Van Bodegom P, Schrama M, Boelee E. Creating the Dutch One Health Shared Socio-economic Pathways (SSPs). Reg Environ Change. 2024 Mar;24(1):16.

113. Havelaar AH, Van Rosse F, Bucura C, Toetenel MA, Haagsma JA, Kurowicka D, et al. Prioritizing Emerging Zoonoses in The Netherlands. Ratner AJ, editor. PLoS ONE. 2010 Nov 15;5(11):e13965.

114. Maas M, Gröne A, Kuiken T, van Schaik G, Roest HIJ, van der Giessen JWB. Implementing wildlife disease surveillance in the Netherlands, a One Health approach. OIE Revue Scientifique et Technique. 2016;35(3):863–74.

115. Braks M, van den Kerkhof J. Westnijlvirus in Nederland: Aanpak surveillance en respons 2021-2023 [Internet]. Rijksinstituut voor Volksgezondheid en Milieu RIVM; 2021. Available from: http://hdl.handle.net/10029/625213

116. Chapman GE, Baylis M, Archer DC. Survey of UK horse owners' knowledge of equine arboviruses and disease vectors. Veterinary Record. 2018 Aug;183(5):159–159.

117. Beck C, Jimenez-Clavero M, Leblond A, Durand B, Nowotny N, Leparc-Goffart I, et al. Flaviviruses in Europe: Complex Circulation Patterns and Their Consequences for the Diagnosis and Control of West Nile Disease. IJERPH. 2013 Nov 12;10(11):6049–83.

118. Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu Virus, Italy, 1996. Emerg Infect Dis. 2013 Feb;19(2):274–7.

119. Simonin Y. Circulation of West Nile Virus and Usutu Virus in Europe: Overview and Challenges. Viruses. 2024 Apr 12;16(4):599.

120. Falcão M, Barros M, Duarte MD, Santos FAD, Fagulha T, Henriques M, et al. Genome Characterization and Spaciotemporal Dispersal Analysis of Bagaza Virus Detected in Portugal, 2021. Pathogens. 2023 Jan 17;12(2):150.

121. Jeffries CL, Mansfield KL, Phipps LP, Wakeley PR, Mearns R, Schock A, et al. Louping ill virus: an endemic tick-borne disease of Great Britain. Journal of General Virology. 2014 May 1;95(5):1005–14.

122. Van Heuverswyn J, Hallmaier-Wacker LK, Beauté J, Gomes Dias J, Haussig JM, Busch K, et al. Spatiotemporal spread of tick-borne encephalitis in the EU/EEA, 2012 to 2020. Eurosurveillance. 2023 Mar 16;28(11).

123. Zaaijer HL, Slot E, Molier M, Reusken CBEM, Koppelman MHGM. Usutu virus infection in Dutch blood donors. Transfusion. 2019 Sep;59(9):2931–7.

124. Dekker M, Laverman GD, de Vries A, Reimerink J, Geeraedts F. Emergence of tick-borne encephalitis (TBE) in the Netherlands. Ticks and Tick-borne Diseases. 2019 Jan;10(1):176–9.

125. RIVM. Patient infected with West Nile virus in the Arnhem region [Internet]. 2020 [cited 2024 Apr 26]. Available from: https://www.rivm.nl/en/news/patient-infected-with-west-nile-virus-in-arnhem-region

126. RIVM. Grey heron infected with West Nile virus. National Institute for Public Health and the Environment [Internet]. 2022 Nov 1 [cited 2023 May 3]; Available from: https://www.rivm.nl/node/210071

127. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic Relationships between Flaviviruses as Determined by Cross-neutralization Tests with Polyclonal Antisera. Journal of General Virology. 1989 Jan 1;70(1):37–43.

128. Cavalleri JV, Korbacska-Kutasi O, Leblond A, Paillot R, Pusterla N, Steinmann E, et al. European College of Equine Internal Medicine consensus statement on equine flaviviridae infections in Europe. Veterinary Internal Medicne. 2022 Nov;36(6):1858–71.

129. Llorente F, García-Irazábal A, Pérez-Ramírez E, Cano-Gómez C, Sarasa M, Vázquez A, et al. Influence of flavivirus co-circulation in serological diagnostics and surveillance: A model of study using West Nile, Usutu and Bagaza viruses. Transbound Emerg Dis. 2019 Sep;66(5):2100–6.

130. Beck C, Lowenski S, Durand B, Bahuon C, Zientara S, Lecollinet S. Improved reliability of serological tools for the diagnosis of West Nile fever in horses within Europe. Beasley DWC, editor. PLoS Negl Trop Dis. 2017 Sep 15;11(9):e0005936.

131. Adjadj NR, Vervaeke M, Sohier C, Cargnel M, De Regge N. Tick-Borne Encephalitis Virus Prevalence in Sheep, Wild Boar and Ticks in Belgium. Viruses. 2022 Oct 26;14(11):2362.

132. García-Bocanegra I, Jurado-Tarifa E, Cano-Terriza D, Martínez R, Pérez-Marín JE, Lecollinet S. Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis. 2018 Jun;65(3):765–72.

133. Garcia-Vozmediano A, Bellato A, Rossi L, Hoogerwerf MN, Sprong H, Tomassone L. Use of Wild Ungulates as Sentinels of TBEV Circulation in a Naïve Area of the Northwestern Alps, Italy. Life. 2022 Nov 15;12(11):1888.

134. Joó K, Bakonyi T, Szenci O, Sárdi S, Ferenczi E, Barna M, et al. Comparison of assays for the detection of West Nile virus antibodies in equine serum after natural infection or vaccination. Veterinary Immunology and Immunopathology. 2017 Jan;183:1–6.

135. Monaco F, Purpari G, Di Gennaro A, Mira F, Di Marco P, Guercio A, et al. Immunological response in horses following West Nile virus vaccination with inactivated or recombinant vaccine. Veterinaria Italiana. 2019 Mar 31;55(1):73–9.

136. Jahfari S, de Vries A, Rijks JM, Van Gucht S, Vennema H, Sprong H, et al. Tick-Borne Encephalitis Virus in Ticks and Roe Deer, the Netherlands. Emerg Infect Dis. 2017 Jun;23(6):1028–30.

137. Wijburg SR, Fonville M, de Bruin A, van Rijn PA, Montizaan MGE, van den Broek J, et al. Prevalence and predictors of vector-borne pathogens in Dutch roe deer. Parasites Vectors. 2022 Mar 5;15(1):76.

138. KNHS. Netherlands, Land of Horses [Internet]. KNHS; 2016. Available from: https://www.knhs.nl/media/11820/netherlands-land-of-horses-v2.pdf

139. Dibevo. Huisdieren in Nederland, 2021 [Internet]. 2022 [cited 2023 May 26]. Available from: https://dibevo.nl/kenniscentrum/huisdieren-in-nederland

140. Abourashed A, De Best PA, Doornekamp L, Sikkema RS, Van Gorp ECM, Timen A, et al. Development and validation of the MosquitoWise survey to assess perceptions towards mosquitoes and mosquito-borne viruses in Europe. Sci Rep. 2024 Jan 20;14(1):1777.

141. Sergeant. Epitools Epidemiological Calculators [Internet]. 2018. (ESG). Available from: http://epitools.ausvet.com.au

142. RIVM. National Institute for Public Health and the Environment. 2023. Tekenencefalitis (TBE). Available from: https://www.rivm.nl/tekenencefalitis

143. Esser HJ, Lim SM, De Vries A, Sprong H, Dekker DJ, Pascoe EL, et al. Continued Circulation of Tick-Borne Encephalitis Virus Variants and Detection of Novel Transmission Foci, the Netherlands. Emerg Infect Dis. 2022 Dec;28(12):2416–24.

144. CBS. Population dashboard: Age distribution [Internet]. 2022 [cited 2023 Aug 24]. Available from: https://www.cbs.nl/en-gb/visualisations/dashboard-population/age/age-distribution

145. CBS. Dashboard Arbeidsmarkt Zorg en Welzijn (AZW) [Internet]. 2022 [cited 2023 Oct 20]. Available from: https://dashboards.cbs.nl/v4/AZWDashboard/

146. Steenbekkers A, Vermeij L, Houwelingen P van. Dorpsleven tussen stad en land: slotpublicatie Sociale Staat van het Platteland. Den Haag: Sociaal en Cultureel Planbureau; 2017.

147. De Bellegarde De Saint Lary C, Kasbergen LMR, Bruijning-Verhagen PCJL, Van Der Jeugd H, Chandler F, Hogema BM, 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 Jun;16:100533.

148. Metz MBC, Olufemi OT, Daly JM, Barba M. Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018. Transbound Emerg Dis. 2021 Jul;68(4):1814–23.

149. Ganzenberg S, Sieg M, Ziegler U, Pfeffer M, Vahlenkamp TW, Hörügel U, et al. Seroprevalence and Risk Factors for Equine West Nile Virus Infections in Eastern Germany, 2020. Viruses. 2022 May 30;14(6):1191.

150. De Heus P, Kolodziejek J, Hubálek Z, Dimmel K, Racher V, Nowotny N, et al. West Nile Virus and Tick-Borne Encephalitis Virus Are Endemic in Equids in Eastern Austria. Viruses. 2021 Sep 19;13(9):1873.

151. Guerrero-Carvajal F, Bravo-Barriga D, Martín-Cuervo M, Aguilera-Sepúlveda P, Ferraguti M, Jiménez-Clavero MÁ, et al. Serological evidence of co-circulation of West Nile and Usutu viruses in equids from western Spain. Transbound Emerg Dis. 2021 May;68(3):1432–44.

152. Magallanes S, Llorente F, Ruiz-López MJ, Martínez-de La Puente J, Soriguer R, Calderon J, et al. Long-term serological surveillance for West Nile and Usutu virus in horses in south-West Spain. One Health. 2023 Dec;17:100578.

153. Klaus C, Ziegler U, Kalthoff D, Hoffmann B, Beer M. Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera. BMC Vet Res. 2014;10(1):78.

154. Percivalle, Cassaniti, Sarasini, Rovida, Adzasehoun, Colombini, et al. West Nile or Usutu Virus? A Three-Year Follow-Up of Humoral and Cellular Response in a Group of Asymptomatic Blood Donors. Viruses. 2020 Jan 29;12(2):157.

155. Trachsel DS, Drozdzewska K, Bergmann F, Ziegler U, Gehlen H. Bestätigte West-Nil-Virus-Infektion bei einem Pferd mit minimalen neurologischen Symptomen und günstigem klinischem Verlauf. Tierarztl Prax Ausg G Grosstiere Nutztiere. 2021 Aug;49(04):281–6.

156. Benzarti E, Linden A, Desmecht D, Garigliany M. Mosquito-borne epornitic flaviviruses: an update and review. Journal of General Virology. 2019 Feb 1;100(2):119–32.

157. Hyde J, Nettleton P, Marriott L, Willoughby K. Louping ill in horses. Veterinary Record. 2007 Apr;160(15):532–532.

158. Timoney PJ, Donnelly WJC, Clements LO, Fenlon M. Encephalitis Caused by Louping ill Virus in a Group of Horses in Ireland. Equine Veterinary Journal. 1976 Jul;8(3):113–7.

159. Pfister K, Armstrong R. Systemically and cutaneously distributed ectoparasiticides: a review of the efficacy against ticks and fleas on dogs. Parasites Vectors. 2016 Dec;9(1):436.

160. Boden LA, Parkin TD, Yates J, Mellor D, Kao RR. An online survey of horse-owners in Great Britain. BMC Vet Res. 2013;9(1):188.

161. Manyweathers J, Field H, Longnecker N, Agho K, Smith C, Taylor M. "Why won't they just vaccinate?" Horse owner risk perception and uptake of the Hendra virus vaccine. BMC Vet Res. 2017 Dec;13(1):103.

162. Adouchief S, Smura T, Sane J, Vapalahti O, Kurkela S. Sindbis virus as a human pathogen-epidemiology, clinical picture and pathogenesis. Rev Med Virol. 2016 Jul;26(4):221–41.

163. ECDC. European Center for Disease Prevention and Control (ECDC). 2023 [cited 2024 Jun 7]. Facts about Sindbis fever. Available from: https://www.ecdc.europa.eu/en/sindbis-fever/facts

164. Storm N, Weyer J, Markotter W, Kemp A, Leman PA, Dermaux-Msimang V, et al. Human cases of Sindbis fever in South Africa, 2006–2010. Epidemiol Infect. 2014 Feb;142(2):234–8.

165. Fourie I, Snyman J, Williams J, Ismail A, Jansen Van Vuren P, Venter M. Epidemiological and Genomic Characterisation of Middelburg and Sindbis Alphaviruses Identified in Horses with Febrile and Neurological Infections, South Africa (2014–2018). Viruses. 2022 Sep 11;14(9):2013.

166. Streng K, Hakze-van der Honing RW, Graham H, Van Oort S, De Best PA, Abourashed A, et al. Orthoflavivirus surveillance in the Netherlands: Insights

from a serosurvey in horses & dogs and a questionnaire among horse owners. Zoonoses and Public Health. 2024 Jul 26;zph.13171.

167. Hesson JC, Lundström JO, Tok A, Östman Ö, Lundkvist Å. Temporal Variation in Sindbis Virus Antibody Prevalence in Bird Hosts in an Endemic Area in Sweden. Coffey LL, editor. PLoS ONE. 2016 Aug 31;11(8):e0162005.

168. Van Vliet J, Musters CJM, Ter Keurs WJ. Changes in migration behaviour of Blackbirds *Turdus merula* from the Netherlands. Bird Study. 2009 Jul;56(2):276–81.

169. Blom R, Schrama MJJ, Spitzen J, Weller BFM, Van Der Linden A, Sikkema RS, et al. Arbovirus persistence in North-Western Europe: Are mosquitoes the only overwintering pathway? One Health. 2023 Jun;16:100467.

170. Reusken C, Vries AD, Ceelen E, Beeuwkes J, Scholte EJ. 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.” European Mosquito Bulletin. 2011;29:66–81.

171. Jöst H, Bialonski A, Storch V, Günther S, Becker N, Schmidt-Chanasit J. Isolation and Phylogenetic Analysis of Sindbis Viruses from Mosquitoes in Germany. J Clin Microbiol. 2010 May;48(5):1900–3.

172. Rau J, Köchling K, Schäfer M, Tews BA, Wylezich C, Schaub GA, et al. Viral RNA in Mosquitoes (Diptera: Culicidae) Collected between 2019 and 2021 in Germany. Viruses. 2023 Nov 23;15(12):2298.

173. Becker N, Petrić D, Zgomba M, Boase C, Madon MB, Dahl C, et al. Mosquitoes: Identification, Ecology and Control. Cham: Springer International Publishing; 2020. (Fascinating Life Sciences).

174. Sane J, Kurkela S, Levanov L, Nikkari S, Vaheri A, Vapalahti O. Development and evaluation of a real-time RT-PCR assay for Sindbis virus detection. Journal of Virological Methods. 2012 Jan;179(1):185–8.

175. Larsson A. AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics. 2014 Nov 15;30(22):3276–8.

176. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 2016 Jul 8;44(W1):W232–5.

177. R Core Team. R: A language and environment for statistical computing. [Internet]. Vienna, Austria.: R Foundation for Statistical Computing; 2023. Available from: https://www.R-project.org/

178. Wickham H. ggplot2: Elegant Graphics for Data Analysis. 2nd ed. 2016. Cham: Springer International Publishing : Imprint: Springer; 2016. 1 p. (Use R!).

179. Yu G. Using ggtree to Visualize Data on Tree-Like Structures. CP in Bioinformatics. 2020 Mar;69(1):e96.

180. Nikolay B. A review of West Nile and Usutu virus co-circulation in Europe: how much do transmission cycles overlap? Trans R Soc Trop Med Hyg. 2015 Oct;109(10):609–18.

181. Piantadosi A, Kanjilal S. Diagnostic Approach for Arboviral Infections in the United States. Kraft CS, editor. J Clin Microbiol. 2020 Nov 18;58(12):e01926-19.

182. Glemain B, De Lamballerie X, Zins M, Severi G, Touvier M, Deleuze JF, et al. Estimating SARS-CoV-2 infection probabilities with serological data and a Bayesian mixture model. Sci Rep. 2024 Apr 25;14(1):9503.

183. Hozé N, Diarra I, Sangaré AK, Pastorino B, Pezzi L, Kouriba B, et al. Model-based assessment of Chikungunya and O'nyong-nyong virus circulation in Mali in a serological cross-reactivity context. Nat Commun. 2021 Nov 18;12(1):6735.

184. Kucharski AJ, Lessler J, Cummings DAT, Riley S. Timescales of influenza A/H3N2 antibody dynamics. Rowland-Jones S, editor. PLoS Biol. 2018 Aug 20;16(8):e2004974.

185. Gutiérrez-Guzmán AV, Vicente J, Sobrino R, Perez-Ramírez E, Llorente F, Höfle U. Antibodies to West Nile virus and related flaviviruses in wild boar, red foxes and other mesomammals from Spain. Veterinary Microbiology. 2012 Oct;159(3–4):291–7.

186. Petruccelli A, Zottola T, Ferrara G, Iovane V, Di Russo C, Pagnini U, et al. West Nile Virus and Related Flavivirus in European Wild Boar (Sus scrofa), Latium Region, Italy: A Retrospective Study. Animals. 2020 Mar 16;10(3):494.

187. Wageningen University & Research. Monitoring programme wild boar [Internet]. 2024 [cited 2024 Mar 25]. Available from: https://www.wur.nl/nl/onderzoek-resultaten/onderzoeksinstituten/bioveterinary-research/diagnostiek/wilde-dieren/monitoringsprogramma-wilde-zwijnen.htm

188. ECDC. West Nile virus infection. Annual Epidemiological Report for 2019. [Internet]. Stockholm: European Centre for Disease Prevention and Control; 2021. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/AER-WNV-infection-2019.pdf

189. Vehtari A, Gelman A, Gabry J. Practical Bayesian model evaluation using leave-one-out cross-validation and WAIC. Stat Comput. 2017 Sep;27(5):1413–32.

190. Vehtari A, Gabry J, Magnusson M, Yao Y, Bürkner PC, Paananen T, et al. loo: Efficient leave-one-out cross-validation and WAIC for Bayesian models [Internet]. 2024. Available from: https://mc-stan.org/loo/

191. De Freitas Costa E, Streng K, Avelino De Souza Santos M, Counotte MJ. The effect of temperature on the boundary conditions of West Nile virus circulation in Europe. Liang S, editor. PLoS Negl Trop Dis. 2024 May 6;18(5):e0012162.

192. Münger E. Emergence and Dynamics of Usutu and West Nile Viruses in the Netherlands, 2016-2022. in preparation;

193. Kostoulas P, Nielsen SS, Branscum AJ, Johnson WO, Dendukuri N, Dhand NK, et al. STARD-BLCM: Standards for the Reporting of Diagnostic accuracy studies that use Bayesian Latent Class Models. Preventive Veterinary Medicine. 2017 Mar;138:37–47.

194. Bergmann F, Schmoock-Wellhausen M, Fast C, Holicki CM, Michel F, Wysocki P, et al. Longitudinal Study of the Occurrence of Usutu Virus and West Nile Virus Infections in Birds in a Zoological Garden in Northern Germany. Pathogens. 2023 May 23;12(6):753.

195. McKee EM, Walker ED, Anderson TK, Kitron UD, Brawn JD, Krebs BL, et al. West Nile Virus antibody decay rate in free-ranging birds. Journal of Wildlife Diseases. 2015 Jul 1;51(3):601.

196. Streng K, de Best PA, Timen A, Koopmans MPG, van der Poel WHM, Sikkema RS. Rapid response screening for emerging zoonotic pathogens, barriers and

opportunities: A study for enhanced preparedness of the Netherlands. One Health. 2023 Jun;16:100507.

197. Walker PJ, Siddell SG, Lefkowitz EJ, Mushegian AR, Adriaenssens EM, Alfenas-Zerbini P, et al. Recent changes to virus taxonomy ratified by the International Committee on Taxonomy of Viruses (2022). Arch Virol. 2022 Nov;167(11):2429–40.

198. Vilibic-Cavlek T, Petrovic T, Savic V, Barbic L, Tabain I, Stevanovic V, et al. Epidemiology of Usutu Virus: The European Scenario. Pathogens. 2020 Aug 26;9(9):699.

199. Vogels CB, Göertz GP, Pijlman GP, Koenraadt CJ. Vector competence of European mosquitoes for West Nile virus. Emerging Microbes & Infections. 2017 Jan;6(1):1–13.

200. Ibáñez Justicia A, Stroo A, Dik M, Beeuwkes J, Scholte EJ. National Mosquito (Diptera: Culicidae) Survey in The Netherlands 2010-2013. Journal of Medical Entomology. 2015 Mar 1;52(2):185–98.

201. Möhlmann TWR, Wennergren U, Tälle M, Favia G, Damiani C, Bracchetti L, et al. Community analysis of the abundance and diversity of mosquito species (Diptera: Culicidae) in three European countries at different latitudes. Parasites Vectors. 2017 Dec;10(1):510.

202. Brugman V, Hernández-Triana L, Medlock J, Fooks A, Carpenter S, Johnson N. The Role of *Culex pipiens* L. (Diptera: Culicidae) in Virus Transmission in Europe. IJERPH. 2018 Feb 23;15(2):389.

203. Roesch F, Fajardo A, Moratorio G, Vignuzzi M. Usutu Virus: An Arbovirus on the Rise. Viruses. 2019;11(640):1–14.

204. Fay RL, Keyel AC, Ciota AT. West Nile virus and climate change. In: Advances in Virus Research [Internet]. Elsevier; 2022 [cited 2024 Apr 16]. p. 147–93. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0065352722000215

205. Ziegler U, Lühken R, Keller M, Cadar D, Van Der Grinten E, Michel F, et al. West Nile virus epizootic in Germany, 2018. Antiviral Research. 2019 Feb;162:39–43.

206. Braks MAH, van den Kerkhof HHTC. Westnijlvirus in Nederland: Aanpak Surveillance en respons 2021-2023. 2021.

207. Vlaskamp DRM, Thijsen SFT, Reimerink J, Hilkens P, Bouvy WH, Bantjes SE, et al. First autochthonous human west nile virus infections in the Netherlands, July to August 2020. Eurosurveillance. 2020;25(46):1–4.

208. Kwan JL, Kluh S, Madon MB, Nguyen D V., Barker CM, Reisen WK. Sentinel chicken seroconversions track tangential transmission of West Nile virus to humans in the greater Los Angeles area of California. American Journal of Tropical Medicine and Hygiene. 2010;83(5):1137–45.

209. Chaintoutis SC, Gewehr S, Mourelatos S, Dovas CI. Serological monitoring of backyard chickens in Central Macedonia-Greece can detect low transmission of West Nile virus in the absence of human neuroinvasive disease cases. Acta Tropica. 2016 Nov;163:26–31.

210. Langevin SA, Bunning M, Davis B, Komar N. Experimental infection of chickens as candidate sentinels for West Nile virus. Emerg Infect Dis. 2001;7(4):726–9.

211. Holicki CM, Michel F, Vasić A, Fast C, Eiden M, Răileanu C, et al. Pathogenicity of West Nile Virus Lineage 1 to German Poultry. Vaccines. 2020 Sep 5;8(3):507.

212. VSKN. Association for Dutch City- and Petting Zoos. 2023 [cited 2023 Oct 3]. Vereniging Stads- en Kinderboerderijen Nederland. Available from: https://www.vskbn.nl/

213. Reisen WK. Landscape Epidemiology of Vector-Borne Diseases. Annu Rev Entomol. 2010 Jan 1;55(1):461–83.

214. Cleton NB, Van Maanen K, Bergervoet SA, Bon N, Beck C, Godeke GJ, et al. A Serological Protein Microarray for Detection of Multiple Cross-Reactive Flavivirus Infections in Horses for Veterinary and Public Health Surveillance. Transbound Emerg Dis. 2017 Dec;64(6):1801–12.

215. Koopmans M, De Bruin E, Godeke GJ, Friesema I, Gageldonk RV, Schipper M, et al. Profiling of humoral immune responses to influenza viruses by using protein microarray. Clinical Microbiology and Infection. 2012 Aug;18(8):797–807.

216. Reusken C, Boonstra M, Rugebregt S, Scherbeijn S, Chandler F, Avšič-Županc T, et al. An evaluation of serological methods to diagnose tick-borne encephalitis

from serum and cerebrospinal fluid. Journal of Clinical Virology. 2019 Nov;120:78–83.

217. RStudio Team. RStudio: Integrated Development for R [Internet]. Boston, MA: RStudio, PBC; 2023. Available from: www.RStudio.com

218. QGIS Development Team. QGIS Geographic Information System. 2021.

219. Blom R, Krol L, Langezaal M, Schrama M, Trimbos KB, Wassenaar D, et al. Blood-feeding patterns of *Culex pipiens* biotype *pipiens* and *pipiens/molestus* hybrids in relation to avian community composition in urban habitats. Parasites & Vectors. 2024;17(94).

220. SOVON. Monitoring dode vogels [Internet]. 2021 [cited 2024 Feb 29]. Available from: https://portal.sovon.nl/dood/result/index/11870?jaar=2021&maand=-1&oorzaak=&pid=0

221. Meister T, Lussy H, Bakonyi T, Šikutová S, Rudolf I, Vogl W, et al. Serological evidence of continuing high Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in Austria. Veterinary Microbiology. 2008 Mar 18;127(3–4):237–48.

222. Nemeth NM, Kratz GE, Bates R, Scherpelz JA, Bowen RA, Komar N. Naturally Induced Humoral Immunity to West Nile Virus Infection in Raptors. EcoHealth. 2008 Sep;5(3):298–304.

223. Nemeth NM, Oesterle PT, Bowen RA. Humoral Immunity to West Nile Virus Is Long-Lasting and Protective in the House Sparrow (*Passer domesticus*). The American Journal of Tropical Medicine and Hygiene. 2009 May 1;80(5):864–9.

224. Santos PD, Michel F, Wylezich C, Höper D, Keller M, Holicki CM, et al. Co-infections: Simultaneous detections of West Nile virus and Usutu virus in birds from Germany. Transbounding Emerging Dis. 2022 Mar;69(2):776–92.

225. Kuno G. Persistence of arboviruses and antiviral antibodies in vertebrate hosts: its occurrence and impacts. Rev Med Virol. 2001 May;11(3):165–90.

226. Nemeth N, Young G, Ndaluka C, Bielefeldt-Ohmann H, Komar N, Bowen R. Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). Arch Virol. 2009 May;154(5):783–9.

227. Jansen S, Heitmann A, Lühken R, Leggewie M, Helms M, Badusche M, et al. *Culex torrentium*: A Potent Vector for the Transmission of West Nile Virus in Central Europe. Viruses. 2019 May 29;11(6):492.

228. Lim SM, Brault AC, Amerongen G Van, Sewbalaksing VD. Susceptibility of European jackdaws (Corvus monedula) to experimental infection with lineage 1 and 2 West Nile viruses. Journal of General Virologyn Virol. 2014;95(0 6):1320–9.

229. Wet Dieren (Animal Health Law), article 5.4. [Internet]. 2011. Available from: https://wetten.overheid.nl/jci1.3:c:BWBR0041794&z=2022-01-18&g=2022-01-18

230. OIE Terrestrial Animal Health Code (Online Access) glossary [Internet]. World Organisation for Animal Health (WOAH); 2021. Available from: https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/?id=169&L=1&htmfile=glossaire.htm#terme_services_veterinaires

231. Gezondheidswet (Public Health Law), chapter 4. [Internet]. 1956. Available from: https://wetten.overheid.nl/jci1.3:c:BWBR0002202&z=2021-07-01&g=2021-07-01

232. WOAH. Animal Health Surveillance. In: Terrestrial Animal Health Code, Chapter 14 [Internet]. Office International des Epizooties; 2021. p. 1–10. Available from: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_surveillance_general.pdf

233. Olson SH, Reed P, Cameron KN, Ssebide BJ, Johnson CK, Morse SS, et al. Dead or alive: Animal sampling during Ebola hemorrhagic fever outbreaks in humans. Emerging Health Threats Journal. 2012;5(1).

234. WOAH. Considerations on monitoring SARS-CoV-2 in animals [Internet]. World Organisation for Animal Health (WOAH); 2022. p. 1–12. Available from: https://www.oie.int/app/uploads/2022/02/en-sars-cov-2-surveillance-.pdf

235. WOAH. Joint statement on the prioritization of monitoring SARS-CoV-2 infection in wildlife and preventing the formation of animal reservoirs [Internet]. World Organisation for Animal Health (WOAH); 2022. Available from: https://www.oie.int/en/joint-statement-on-the-prioritization-of-monitoring-sars-cov-2-infection-in-wildlife-and-preventing-the-formation-of-animal-reservoirs/

236. WHO. Protocol for the investigation of acute respiratory illness outbreaks of unknown etiology [Internet]. Brazzaville, Congo: World Health Organization Regional Office for Africa; 2016. Available from: https://afro.who.int/sites/default/files/2017-06/protocol-for-the-investigation-of-acute-respiratory-illness-outbreaks-of....pdf

237. WOAH, FAO. A Field Manual for Animal Disease Outbreak Investigation and Management [Internet]. South-East Asia and China Foot-and-Mouth Disease (SEACFMD) Campaign; 2018. p. 1–89. Available from: https://rr-asia.oie.int/wp-content/uploads/2020/02/manual_outbreak-investigation.pdf

238. Johnson I, Hansen A, Bi P. The challenges of implementing an integrated One Health surveillance system in Australia. Zoonoses and Public Health. 2018;65(1):e229–36.

239. Li H, Chen Y, Machalaba CC, Tang H, Chmura AA, Fielder MD, et al. Wild animal and zoonotic disease risk management and regulation in China: Examining gaps and One Health opportunities in scope, mandates, and monitoring systems. One Health. 2021;13(June):100301.

240. Mueni Katee S, Keambou Tiambo C. Discussing the Drawbacks of the Implementation of Access and Benefit Sharing of the Nagoya Protocol Following the COVID-19 Pandemic. Front Public Health. 2021 Dec 10;9:639581.

241. Ribeiro C dos S, Roode MY van, Haringhuizen GB, Koopmans MP, Claassen E, Burgwal LHM van de. How ownership rights over microorganisms affect infectious disease control and innovation: A root-cause analysis of barriers to data sharing as experienced by key stakeholders. PLoS ONE. 2018;13(5):1–19.

242. Schwind JS, Goldstein T, Thomas K, Mazet JA, Smith WA. Capacity building efforts and perceptions for wildlife surveillance to detect zoonotic pathogens: comparing stakeholder perspectives. BMC Public Health. 2014 Dec;14(1):684.

243. Uchtmann N, Herrmann JA, Hahn EC, Beasley VR. Barriers to, Efforts in, and Optimization of Integrated One Health Surveillance: A Review and Synthesis. EcoHealth. 2015;12(2):368–84.

244. Sharun K, Dhama K, Pawde AM, Gortázar C, Tiwari R, Bonilla-Aldana DK, et al. SARS-CoV-2 in animals: potential for unknown reservoir hosts and public health implications. The veterinary quarterly. 2021 Dec;41(1):181–201.

245. Chandler JC, Bevins SN, Ellis JW, Linder TJ, Tell RM, Jenkins-Moore M, et al. SARS-CoV-2 exposure in wild white-tailed deer (*Odocoileus virginianus*). Proceedings of the National Academy of Sciences. 2021;118(47):e2114828118.

246. Hale VL, Dennis PM, McBride DS, Nolting JM, Madden C, Huey D, et al. SARS-CoV-2 infection in free-ranging white-tailed deer. Nature. 2022 Feb;602(7897):481–6.

247. Pickering B, Lung O, Maguire F, Kruczkiewicz P, Kotwa JD, Buchanan T, et al. Divergent SARS-CoV-2 variant emerges in white-tailed deer with deer-to-human transmission. Nature Microbiology. 2022 Nov;2022.02.22.481551.

248. Colella JP, Bates J, Burneo SF, Camacho MA, Bonilla CC, Constable I, et al. Leveraging natural history biorepositories as a global, decentralized, pathogen surveillance network. Vol. 17, PLoS Pathogens. Public Library of Science; 2021.

249. WHO. WHO-convened Global Study of Origins of SARS-CoV-2: China Part [Internet]. World Health Organization (WHO); 2021. Available from: https://www.who.int/publications/i/item/who-convened-global-study-of-origins-of-sars-cov-2-china-part

250. Morens DM, Fauci AS. Emerging Pandemic Diseases: How We Got to COVID-19. Cell. 2020;182(5):1077–92.

251. Jacobs P, Kessel R van, Rosa M de, Fitz-James IS, Verbart C. Vademecum Zoönosen [Internet]. RIVM. 2021. Available from: https://www.rivm.nl/vademecum-zoonosen

252. Augustijn-Schretlen M, van Klink E, Tobias T, van der Giessen JWB. Early warning and surveillance systems in the Netherlands. Farm animals and horses. 2020.

253. AVINED. Monitoring Pluimvee. https://www.avined.nl/themas/pluimveegezondheid/monitoring-pluimveeziekten.

254. CBS. Landbouw; gewassen, dieren, grondgebruik en arbeid op nationaal niveau. https://opendata.cbs.nl/statline/#/CBS/nl/dataset/81302ned/table?fromstatweb. Central Statistical Office (CBS);

255. Uitvoeringswet Algemene verordening gegevensbescherming (GDPR), article 22. [Internet]. 2018. Available from:

19/10/2022https://wetten.overheid.nl/jci1.3:c:BWBR0040940&hoofdstuk=3&paragraaf=3.1&artikel=22&z=2018-05-25&g=2018-05-25

256. Wet Dieren (Animal Health Law), article 2.12. [Internet]. 2011. Available from: http://wetten.overheid.nl/jci1.3:c:BWBR0030250&hoofdstuk=2&paragraaf=1&artikel=2.12

257. Working document on specific articles in Directive 2010/63/EU [Internet]. European Union; 2011. p. 1–12. Available from: https://ec.europa.eu/environment/chemicals/lab_animals/pdf/Consensus_document.pdf

258. Wet op de Dierproeven (Animal Research Act), article 1. [Internet]. 1977. Available from: https://wetten.overheid.nl/jci1.3:c:BWBR0003081&paragraaf=1&z=2021-07-01&g=2021-07-01

259. Commission Implementing Regulation (EU) 2020/2002 [Internet]. 2020. Available from: http://data.europa.eu/eli/reg_impl/2020/2002/oj

260. Wet Dieren (Animal Health Law), article 5.3. [Internet]. 2011. Available from: https://wetten.overheid.nl/jci1.3:c:BWBR0030250&hoofdstuk=5&paragraaf=2&artikel=5.3&z=2022-01-28&g=2022-01-28

261. Hubbard LE, Kolpin DW, Fields CL, Hladik ML, Iwanowicz LR. Highlighting the complexities of a groundwater pilot study during an avian influenza outbreak: Methods, lessons learned, and select contaminant results. Environmental Research. 2017;158:212–24.

262. Schemann K, Gillespie JA, Toribio JALML, Ward MP, Dhand NK. Controlling equine influenza: policy networks and decision-making during the 2007 Australian equine influenza outbreak. Transboundary and emerging diseases. 2014;61(5):449–63.

263. Bisdorff B, Schauer B, Taylor N, Rodríguez-Prieto V, Comin A, Brouwer A, et al. Active animal health surveillance in European Union Member States: Gaps and opportunities. Epidemiology and Infection. 2017;145(4):802–17.

264. Edelstein M, Lee LM, Herten-Crabb A, Heymann DL, Harper DR. Strengthening Global Public Health Surveillance through Data and Benefit Sharing. Emerging Infectious Diseases. 2018 Jul;24(7):1324–30.

265. Rubin C, Dunham B, Sleeman J. Making One Health a Reality–Crossing Bureaucratic Boundaries. Microbiology spectrum. 2014 Feb;2(1):OH-0016-2012.

266. Sok J, Horne P van, Meuwissen M. The impact of the fipronil crisis on the financial performance of Dutch laying hen farms. Parasites & Vectors. 2020;13(1):589.

267. Roode M van, Farag E, Ahmed M, Moustafa A, Claassen E, Nour M, et al. Data sharing in public health emergencies: Analysis of barriers and enablers from an outbreak response perspective (SHARE). The case of Middle East Respiratory Syndrome (MERS). 2018.

268. Thomas LF, Rushton J, Bukachi SA, Falzon LC, Howland O, Fèvre EM. Cross-Sectoral Zoonotic Disease Surveillance in Western Kenya: Identifying Drivers and Barriers Within a Resource Constrained Setting. Frontiers in Veterinary Science. 2021 Jun;8.

269. Research WU&. Prevalentieonderzoek Brucella canis [Internet]. 2020. Available from: https://www.wur.nl/nl/onderzoek-resultaten/onderzoeksprojecten-lnv/expertisegebieden/kennisonline/prevalentieonderzoek-brucella-canis.htm

270. Tacconelli E, Gorska A, Carrara E, Davis RJ, Bonten M, Friedrich AW, et al. Challenges of data sharing in European Covid-19 projects: A learning opportunity for advancing pandemic preparedness and response. The Lancet Regional Health - Europe. 2022 Oct;21:100467.

271. Müller MA, Corman VM, Jores J, Meyer B, Younan M, Liljander A, et al. MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983-1997. Emerging infectious diseases. 2014 Dec;20(12):2093–5.

272. Daubney R, Hudson JR, Garnham PC. Enzootic hepatitis or rift valley fever. An undescribed virus disease of sheep cattle and man from east africa. J Pathol. 1931 Jan;34(4):545–79.

273. Jonkmans N, D'Acremont V, Flahault A. Scoping future outbreaks: a scoping review on the outbreak prediction of the WHO Blueprint list of priority diseases. BMJ Glob Health. 2021 Sep;6(9):e006623.

274. EFSA. Assessment of the control measures of the category A diseases of Animal Health Law: Rift Valley Fever. EFSA Journal. 2022 Jan;20(1).

275. Wright D, Kortekaas J, Bowden TA, Warimwe GM. Rift Valley fever: biology and epidemiology. Journal of General Virology. 2019 Aug 1;100(8):1187–99.

276. WOAH. Technical Disease Card: Rift Valley Fever [Internet]. 2019. Available from: https://www.woah.org/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/RIFT_VALLEY_FEVER.pdf

277. Botros B, Omar A, Elian K, Mohamed G, Soliman A, Salib A, et al. Adverse response of non-indigenous cattle of European breeds to live attenuated Smithburn Rift Valley fever vaccine. Journal of Medical Virology. 2006 Jun;78(6):787–91.

278. Leroux-Roels I, Prajeeth CK, Aregay A, Nair N, Rimmelzwaan GF, Osterhaus ADME, et al. Safety and immunogenicity of the live-attenuated hRVFV-4s vaccine against Rift Valley fever in healthy adults: a dose-escalation, placebo-controlled, first-in-human, phase 1 randomised clinical trial. The Lancet Infectious Diseases. 2024 Jul;S147330992400375X.

279. Bazanow B, Stygar D, Romuk E, Skrzep-Poloczek B, Pacoń J, Gadzała Ł, et al. Preliminary serological investigation of Rift Valley fever in Poland. J Vector Borne Dis. 2018;55(4):324.

280. García-Bocanegra I, Paniagua J, Cano-Terriza D, Arenas-Montes A, Fernández-Morente M, Napp S. Absence of Rift Valley fever virus in domestic and wild ruminants from Spain. Veterinary Record. 2016 Jul;179(2):48–48.

281. Chevalier V. Relevance of Rift Valley fever to public health in the European Union. Clinical Microbiology and Infection. 2013 Aug;19(8):705–8.

282. Chevalier V, Pépin M, Plée L, Lancelot R. Rift Valley fever - a threat for Europe? Eurosurveillance [Internet]. 2010 Mar 11 [cited 2024 Aug 2];15(10). Available from: https://www.eurosurveillance.org/content/10.2807/ese.15.10.19506-en

283. EFSA. Rift Valley Fever – epidemiological update and risk of introduction into Europe. EFSA Journal [Internet]. 2020 Mar [cited 2023 Jun 5];18(3). Available from: https://data.europa.eu/doi/10.2903/j.efsa.2020.6041

284. Dufour B, Moutou F, Hattenberger AM, Rodhain F. Global change: impact, management, risk approach and health measures – the case of Europe. Revue Scientifique et Technique de l'OIE. 2008;27(2):541–50.

285. Esser HJ, Liefting Y, Ibáñez-Justicia A, Van Der Jeugd H, Van Turnhout CAM, Stroo A, et al. Spatial risk analysis for the introduction and circulation of six arboviruses in the Netherlands. Parasites Vectors. 2020 Dec;13(1):464.

286. Drouin A, Chevalier V, Durand B, Balenghien T. Vector Competence of Mediterranean Mosquitoes for Rift Valley Fever Virus: A Meta-Analysis. Pathogens. 2022 Apr 23;11(5):503.

287. Birnberg L, Talavera S, Aranda C, Núñez AI, Napp S, Busquets N. Field-captured Aedes vexans (Meigen, 1830) is a competent vector for Rift Valley fever phlebovirus in Europe. Parasites Vectors. 2019 Dec;12(1):484.

288. ECDC. *Culex pipiens* - Factsheet for experts. [Internet]. 2020. Available from: https://www.ecdc.europa.eu/en/infectious-disease-topics/related-public-health-topics/disease-vectors/facts/mosquito-factsheets/culex-pipiens

289. KNMI. Temperature - gridded daily mean temperature in the Netherlands [Internet]. 2023. Available from: https://dataplatform.knmi.nl/dataset/tg1-5

290. KNMI. KNMI'23 klimaatscenario's voor Nederland [Internet]. 2023. Available from: https://cdn.knmi.nl/system/data_center_publications/files/000/071/901/original/KNMI23_klimaatscenarios_gebruikersrapport_23-03.pdf

291. Krol L, Dellar M, Ibáñez Justicia A, van der Schrier G, Schrama MJJ, Geerling GJ, et al. Predicting *Culex pipiens* abundances in a changing world: evaluating multiple scenarios for a low lying delta. in preparation;

292. Dellar M, Streng K, Van Bodegom P, Ibáñez Justicia A. Current and future probability of occurrence of the floodwater mosquito Aedes vexans (Meigen, 1830) in the Netherlands. in preparation;

293. Rubel F, Brugger K, Hantel M, Chvala-Mannsberger S, Bakonyi T, Weissenböck H, et al. Explaining Usutu virus dynamics in Austria: Model development and calibration. Preventive Veterinary Medicine. 2008 Jul;85(3–4):166–86.

294. Harrington LC, Fleisher A, Ruiz-Moreno D, Vermeylen F, Wa CV, Poulson RL, et al. Heterogeneous Feeding Patterns of the Dengue Vector, Aedes aegypti, on Individual Human Hosts in Rural Thailand. Bingham A, editor. PLoS Negl Trop Dis. 2014 Aug 7;8(8):e3048.

295. Cecilia H, Métras R, Fall AG, Lo MM, Lancelot R, Ezanno P. It's risky to wander in September: Modelling the epidemic potential of Rift Valley fever in a Sahelian setting. Epidemics. 2020 Dec;33:100409.

296. Ba Y, Diallo D, Dia I, Diallo M. [Feeding pattern of Rift Valley Fever virus vectors in Senegal. Implications in the disease epidemiology]. Bull Soc Pathol Exot. 2006 Oct;99(4):283–9.

297. Shocket MS, Verwillow AB, Numazu MG, Slamani H, Cohen JM, El Moustaid F, et al. Transmission of West Nile and five other temperate mosquito-borne viruses peaks at temperatures between 23°C and 26°C. eLife. 2020 Sep 15;9:e58511.

298. Fischer EA, Boender GJ, Nodelijk G, de Koeijer AA, van Roermund HJ. The transmission potential of Rift Valley fever virus among livestock in the Netherlands: a modelling study. Vet Res. 2013;44(1):58.

299. CRV. Bedrijven en koeien in cijfers - Nederland [Internet]. 2024. Available from: https://www.cooperatie-crv.nl/downloads/stamboek/bedrijven-en-koeien-in-cijfers/

300. Gaff HD, Hartley DM, Leahy NP. An Epidemiological Model of Rift Valley Fever. Electronic Journal of Differential Equations. 2007;2007(115):1–12.

301. Diekmann O, Heesterbeek JAP, Roberts MG. The construction of next-generation matrices for compartmental epidemic models. J R Soc Interface. 2010 Jun 6;7(47):873–85.

302. Dorland R van, Beersma J, Bessembinder J, Bloemendaal N, Brink H van den, Blanes MB, et al. KNMI National Climate Scenarios 2023 for the Netherlands. Koninklijk Nederlands Meteorologisch Instituut (KNMI); 2023 p. 365. Report No.: WR-23-02.

303. Dellar M, Geerling GJ, Kok K, van Bodegom P, van der Schrier G, Schrama M, et al. Future land use maps for the Netherlands based on the Dutch One Health Shared Socio-economic Pathways. in preparation.

304. Farooq Z, Sjödin H, Semenza JC, Tozan Y, Sewe MO, Wallin J, et al. European projections of West Nile virus transmission under climate change scenarios. One Health. 2023 Jun;16:100509.

305. Bron GM, Wichgers Schreur PJ, De Jong MCM, Van Keulen L, Vloet RPM, Koenraadt CJM, et al. Quantifying Rift Valley fever virus transmission efficiency in a lamb-mosquito-lamb model. Front Cell Infect Microbiol. 2023 Dec 18;13:1206089.

306. Cecilia H, Drouin A, Métras R, Balenghien T, Durand B, Chevalier V, et al. Mechanistic models of Rift Valley fever virus transmission: A systematic review. Turell MJ, editor. PLoS Negl Trop Dis. 2022 Nov 18;16(11):e0010339.

307. Greenberg JA, Lujan DA, DiMenna MA, Wearing HJ, Hofkin BV. Identification of Blood Meal Sources in *Aedes vexans* and *Culex quinquefasciatus* in Bernalillo County, New Mexico. Journal of Insect Science. 2013 Jul;13(75):1–12.

308. Kurucz K, Kepner A, Krtinić B, Hederics D, Földes F, Brigetta Z, et al. Blood-meal analysis and avian malaria screening of mosquitoes collected from human-inhabited areas in Hungary and Serbia. Journal of the European Mosquito Control Association. 2018;36:3–13.

309. Field EN, Shepard JJ, Clifton ME, Price KJ, Witmier BJ, Johnson K, et al. Semi-field and surveillance data define the natural diapause timeline for Culex pipiens across the United States. Commun Biol. 2022 Nov 27;5(1):1300.

310. Blom R, Spitzen J, de Haan T, Koenraadt CJM. Phenotypical aspects of *Culex pipiens* biotype *pipiens* during diapause: lipid utilization, body size, insemination, and parity. in preparation;

311. Costello RA, Brust RA. Longevity of Aedes vexans Under Different Temperatures and Relative Humidities in the Laboratory12. Journal of Economic Entomology. 1971 Feb 1;64(1):324–5.

312. Gibbs PH, Cannon TL, Romoser WS, Faran ME, Routier RG, Bailey CL, et al. Reduced Survival of Adult *Culex Pipiens* Infected with Rift Valley Fever Virus. The American Journal of Tropical Medicine and Hygiene. 1987 Sep 1;37(2):403–9.

313. van Ewijk C, Feenstra S, ter Bogt-Kappert C, Braks M, Franz E, Geurts van Kessel C. Westnijlvirus in Nederland. Surveillance en Respons 2021-2023 Eindrapport [Internet]. Rijksinstituut voor Volksgezondheid en Milieu RIVM; 2024 [cited 2024 Jul 18]. Available from: https://rivm.openrepository.com/handle/10029/627589

314. Graham H, Streng K, Henneuse H, Fabius L, Holwerda M, van den Wollenberg L, et al. Westnijlvirus dichterbij dan gedacht. Tijdschrift voor Diergeneeskunde. 2024;149(5):36–9.

315. Bergmann F, Trachsel DS, Stoeckle SD, Sierra JB, Lübke S, Groschup MH, et al. Seroepidemiological Survey of West Nile Virus Infections in Horses from Berlin/Brandenburg and North Rhine-Westphalia, Germany. Viruses. 2022 Jan;14(2):243.

316. Ziegler U, Angenvoort J, Klaus C, Nagel-Kohl U, Sauerwald C, Thalheim S, et al. Use of Competition ELISA for Monitoring of West Nile Virus Infections in Horses in Germany. IJERPH. 2013 Jul 24;10(8):3112–20.

317. Roelandt S, Suin V, Stede YV der, Lamoral S, Marche S, Tignon M, et al. First TBEV serological screening in Flemish wild boar. Infection Ecology & Epidemiology. 2016 Jan;6(1):31099.

318. Trozzi G, Adjadj NR, Vervaeke M, Matthijs S, Sohier C, De Regge N. Comparison of Serological Methods for Tick-Borne Encephalitis Virus-Specific Antibody Detection in Wild Boar and Sheep: Impact of the Screening Approach on the Estimated Seroprevalence. Viruses. 2023 Feb 6;15(2):459.

319. Hesson JC, Verner-Carlsson J, Larsson A, Ahmed R, Lundkvist Å, Lundström JO. *Culex torrentium* Mosquito Role as Major Enzootic Vector Defined by Rate of Sindbis Virus Infection, Sweden, 2009. Emerg Infect Dis. 2015 May;21(5):875–8.

320. Mitter H, Techen AK, Sinabell F, Helming K, Schmid E, Bodirsky BL, et al. Shared Socio-economic Pathways for European agriculture and food systems: The Eur-Agri-SSPs. Global Environmental Change. 2020 Nov;65:102159.

321. ILRI. Preventing and controlling human diseases transmitted by animals saves millions of lives and livelihoods. Livestock pathways to 2030: One Health Brief 2. [Internet]. Nairobi: International Livestock Research Institute; 2021. Available from: https://hdl.handle.net/10568/113056

322. Petrović T, Šekler M, Petrić D, Lazić S, Debeljak Z, Vidanović D, et al. Methodology and results of integrated WNV surveillance programmes in Serbia. Forrester N, editor. PLoS ONE. 2018 Apr 6;13(4):e0195439.

323. Riles MT, Martin D, Mulla C, Summers E, Duke L, Clauson J, et al. WEST NILE VIRUS SURVEILLANCE IN SENTINEL CHICKENS AND MOSQUITOES IN PANAMA

CITY BEACH, FLORIDA, FROM 2014 TO 2020. JOURNAL OF THE AMERICAN MOSQUITO CONTROL ASSOCIATION. 2022;38(3).

324. WUR. Agrimatie. 2021 [cited 2024 Sep 28]. Economische intensiteit - Pluimveehouderij. Available from: https://agrimatie.nl/SectorResultaat.aspx?subpubID=2232&sectorID=2249&themaID=2285

325. Corrigan RLA, Waldner C, Epp T, Wright J, Whitehead SM, Bangura H, et al. Prediction of human cases of West Nile virus by equine cases, Saskatchewan, Canada, 2003. Preventive Veterinary Medicine. 2006 Oct;76(3–4):263–72.

326. Young JJ, Coulombier D, Domanović D, European Union West Nile fever working group, Zeller H, Gossner CM. One Health approach for West Nile virus surveillance in the European Union: relevance of equine data for blood safety. Eurosurveillance. 2019 Apr 18;24(16).

327. Elbers ARW, Gorgievski-Duijvesteijn MJ, Van Der Velden PG, Loeffen WLA, Zarafshani K. A socio-psychological investigation into limitations and incentives concerning reporting a clinically suspect situation aimed at improving early detection of classical swine fever outbreaks. Veterinary Microbiology. 2010 Apr;142(1–2):108–18.

328. Elbers ARW, Gorgievski-Duijvestein MJ, Zarafshani K, Kosh G. To report or not to report: a psychological investigation aimed at improving detection of avian influenza. Revue Scientifique et Technique de l'OIE. 2010 Dec;29(3):435–49.

329. Molini U, Franzo G, Bonfini B, De Villiers L, De Villiers M, Khaiseb S, et al. Low Seroprevalence of WNV in Namibian Dogs Suggests a Limited Effectiveness as Sentinels for Infection Monitoring. TropicalMed. 2023 Mar 29;8(4):203.

330. Cannon AiB, Luff JA, Brault AC, MacLachlan NJ, Case JB, Green ENG, et al. Acute Encephalitis, Polyarthritis, and Myocarditis Associated with West Nile Virus Infection in a Dog. Journal of Veterinary Internal Medicine. 2006 Sep;20(5):1219–23.

331. Gonzalo-Nadal V, Kohl A, Rocchi M, Brennan B, Hughes J, Nichols J, et al. Suspected tick-borne flavivirus meningoencephalomyelitis in dogs from the UK: six cases (2021). J of Small Animal Practice. 2024 Feb;65(2):132–43.

332. Lichtensteiger CA, Heinz-Taheny K, Osborne TS, Novak RJ, Lewis BA, Firth ML. West Nile Virus Encephalitis and Myocarditis in Wolf and Dog. Emerg Infect Dis. 2003 Oct;9(10):1303–6.

333. Pfeffer M, Dobler G. Tick-borne encephalitis virus in dogs - is this an issue? Parasites Vectors. 2011 Dec;4(1):59.

334. Sioutas G, Tsakou K, Top C, Jongejan F, Papadopoulos E. First clinical case of tick-borne encephalitis (TBE) in a dog in Greece. Ticks and Tick-borne Diseases. 2023 Nov;14(6):102226.

335. Grassi L, Drigo M, Zelená H, Pasotto D, Cassini R, Mondin A, et al. Wild ungulates as sentinels of flaviviruses and tick-borne zoonotic pathogen circulation: an Italian perspective. BMC Vet Res. 2023 Sep 14;19(1):155.

336. Arenas-Montes A, García-Bocanegra I, Paniagua J, Franco JJ, Miró F, Fernández-Morente M, et al. Blood sampling by puncture in the cavernous sinus from hunted wild boar. Eur J Wildl Res. 2013 Apr;59(2):299–303.

337. Boadella M, Gortázar C. Effect of haemolysis and repeated freeze-thawing cycles on wild boar serum antibody testing by ELISA. BMC Res Notes. 2011 Dec;4(1):498.

338. Bauer BU, Könenkamp L, Stöter M, Wolf A, Ganter M, Steffen I, et al. Increasing awareness for tick-borne encephalitis virus using small ruminants as suitable sentinels: Preliminary observations. One Health. 2021 Jun;12:100227.

339. Gonzalez G, Bournez L, Moraes RA, Marine D, Galon C, Vorimore F, et al. A One-Health Approach to Investigating an Outbreak of Alimentary Tick-Borne Encephalitis in a Non-endemic Area in France (Ain, Eastern France): A Longitudinal Serological Study in Livestock, Detection in Ticks, and the First Tick-Borne Encephalitis Virus Isolation and Molecular Characterisation. Front Microbiol. 2022 Apr 11;13:863725.

340. Frías M, Fischer K, Castro-Scholten S, Bost C, Cano-Terriza D, Risalde MÁ, et al. Epidemiologic Survey of Crimean-Congo Hemorrhagic Fever Virus in Suids, Spain. Emerg Infect Dis. 2024 May;30(5).

341. Juricová Z, Mitterpák J, Prokopic J, Hubálek Z. Circulation of mosquito-borne viruses in large-scale sheep farms in eastern Slovakia. Folia parasitologica. 1986 Feb;33:285–8.

342. Schuster I, Mertens M, Mrenoshki S, Staubach C, Mertens C, Brüning F, et al. Sheep and goats as indicator animals for the circulation of CCHFV in the environment. Exp Appl Acarol. 2016 Mar;68(3):337–46.

343. Selim A, Abdelhady A. The first detection of anti-West Nile virus antibody in domestic ruminants in Egypt. Trop Anim Health Prod. 2020 Nov;52(6):3147–51.

344. Petrović T, Šekler M, Debeljak Z, Petric D, Labus T, Matović K, et al. RESULTS OF WNV MONITORING PROGRAM IN SERBIA IN 2022. Beograd: Srpsko veterinarsko društvo, Sekcija za zoonoze; 2023. 158–161 p.

345. Angeloni G, Bertola M, Lazzaro E, Morini M, Masi G, Sinigaglia A, et al. Epidemiology, surveillance and diagnosis of Usutu virus infection in the EU/EEA, 2012 to 2021. Eurosurveillance. 2023 Aug 17;28(33).

346. Lu L, Zhang F, Oude Munnink BB, Munger E, Sikkema RS, Pappa S, et al. West Nile virus spread in Europe: Phylogeographic pattern analysis and key drivers. Bessen DE, editor. PLoS Pathog. 2024 Jan 25;20(1):e1011880.

347. Bisanzio D, McMillan JR, Barreto JG, Blitvich BJ, Mead DG, O'Connor J, et al. Evidence for West Nile Virus Spillover into the Squirrel Population in Atlanta, Georgia. Vector-Borne and Zoonotic Diseases. 2015 May;15(5):303–10.

348. Camp JV, Desvars-Larrive A, Nowotny N, Walzer C. Monitoring Urban Zoonotic Virus Activity: Are City Rats a Promising Surveillance Tool for Emerging Viruses? Viruses. 2022 Jul 11;14(7):1516.

349. Chen S wei, Jiang L na, Zhong X shan, Zheng X yan, Ma S juan, Xiong Y quan, et al. Serological Prevalence Against Japanese Encephalitis Virus-Serocomplex Flaviviruses in Commensal and Field Rodents in South China. Vector-Borne and Zoonotic Diseases. 2016 Dec;16(12):777–80.

350. Rijks JM, Montizaan MGE, Bakker N, de Vries A, Van Gucht S, Swaan C, et al. Tick-Borne Encephalitis Virus Antibodies in Roe Deer, the Netherlands. Emerg Infect Dis. 2019 Feb;25(2):342–5.

351. Commission implementing decision (EU) 2018/945 - on the communicable diseases and related special health issues to be covered by epidemiological surveillance as well as relevant case definitions. Official Journal of the European Union [Internet]. 2018;L170/1. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945

352. Kasbergen LMR, Nieuwenhuijse DF, De Bruin E, Sikkema RS, Koopmans MPG. The increasing complexity of arbovirus serology: An in-depth systematic review on cross-reactivity. Vasilakis N, editor. PLoS Negl Trop Dis. 2023 Sep 22;17(9):e0011651.

353. Fischer C, Jo WK, Haage V, Moreira-Soto A, De Oliveira Filho EF, Drexler JF. Challenges towards serologic diagnostics of emerging arboviruses. Clinical Microbiology and Infection. 2021 Sep;27(9):1221–9.

354. Crill WD, Chang GJJ. Localization and Characterization of Flavivirus Envelope Glycoprotein Cross-Reactive Epitopes. J Virol. 2004 Dec 15;78(24):13975–86.

355. WHO. Laboratory testing for Zika virus and dengue virus infections. 2022. Report No.: WHO/ZIKV_DENV/LAB/2022.1.

356. Yeh JY, Lee JH, Park JY, Seo HJ, Moon JS, Cho IS, et al. A Diagnostic Algorithm to Serologically Differentiate West Nile Virus from Japanese Encephalitis Virus Infections and Its Validation in Field Surveillance of Poultry and Horses. Vector-Borne and Zoonotic Diseases. 2012 May;12(5):372–9.

357. Calisher CH, Shope RE, Brandt W, Casals J, Karabatsos N, Murphy FA, et al. Proposed Antigenic Classification of Registered Arboviruses I. Togaviridae, *Alphavirus*. Intervirology. 1980;14(5–6):229–32.

358. Zhao X, Siegel K, Chen MI, Cook AR. Rethinking thresholds for serological evidence of influenza virus infection. Influenza Resp Viruses. 2017 May;11(3):202–10.

359. Bordier M, Uea-Anuwong T, Binot A, Hendrikx P, Goutard FL. Characteristics of One Health surveillance systems: A systematic literature review. Preventive Veterinary Medicine. 2020 Aug;181:104560.

360. Schauer B, Martínez-Avilés M, Comin A, Rodríguez-Prieto V, Dorea F, Häsler B, et al. "Surveillance is a public good" – but how public is it. In Copenhagen; 2014. Available from: https://www.fp7-risksur.eu/sites/default/files/documents/publications/EpiZone2014_BSchauer_HowPublicIsSurveillance.pdf

361. Herten J van, Verweij MF, Bovenkerk B. Considerations for an ethic of One Health: towards a socially responsible zoonotic disease control. Wageningen: Wageningen University; 2021.

362. VanDeVeer D. Interspecific Justice. Inquiry. 1979 Jan;22(1–4):55–79.

363. EFSA Panel on Animal Health and Welfare (AHAW), More S, Bøtner A, Butterworth A, Calistri P, Depner K, et al. Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Japanese encephalitis (JE). EFS2 [Internet]. 2017 Jul [cited 2024 Aug 16];15(7). Available from: https://data.europa.eu/doi/10.2903/j.efsa.2017.4948

364. AHA/AUSVETPLAN. Japanese encephalitis [Internet]. Australian Health Agency; 1998. Available from: https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents/

365. Gossner CM, Dhollander S, Presser LD, Briet O, Bakonyi T, Schaffner F, et al. Potential for emergence of Japanese encephalitis in the European Union. Zoonoses and Public Health. 2024 May;71(3):274–80.

366. Godarzi B, Chandler F, Van Der Linden A, Sikkema RS, De Bruin E, Veldhuizen E, et al. A species-independent lateral flow microarray immunoassay to detect WNV and USUV NS1-specific antibodies in serum. One Health. 2024 Jun;18:100668.

367. Kramer LD, Ciota AT. Dissecting vectorial capacity for mosquito-borne viruses. Current Opinion in Virology. 2015 Dec;15:112–8.

368. Boerlijst SP, Johnston ES, Ummels A, Krol L, Boelee E, Van Bodegom PM, et al. Biting the hand that feeds: Anthropogenic drivers interactively make mosquitoes thrive. Science of The Total Environment. 2023 Feb;858:159716.

369. Linthout C, Martins AD, de Wit M, Delecroix C, Abbo SR, Pijlman GP, et al. The potential role of the Asian bush mosquito Aedes japonicus as spillover vector for West Nile virus in the Netherlands. Parasites and Vectors. 2024 Jun 17;17.

370. de Wit MM, Beaunée G, Dellar M, Krol L, Münger E, van der Jeugd H, et al. Hiding in plain sight: characterising the silent reservoir in multi-host disease emergence. in preparation;

371. Dimas Martins A, Ten Bosch Q, Heesterbeek JAP. Exploring the influence of competition on arbovirus invasion risk in communities. Becker D, editor. PLoS ONE. 2022 Oct 12;17(10):e0275687.

372. Krol L, Remmerswaal L, Groen M, Van Der Beek JG, Sikkema RS, Dellar M, et al. Landscape level associations between birds, mosquitoes and microclimates: possible consequences for disease transmission? Parasites Vectors. 2024 Mar 26;17(1):156.

Summary

Mosquito-borne viruses (MBVs) are an emerging threat. Drivers of emergence, such as changes in climate, land use, travel and trade result in an expansion of geographical range of vectors and virus circulation. Many important zoonotic MBVs belong to the genera *Alphavirus* (family *Togaviridae*), *Orthoflavivirus* (family *Flaviviridae*) and *Phlebovirus* (family *Phenuiviridae*). Examples of viruses from these families are Sindbis virus (SINV), West Nile virus (WNV) and Rift Valley Fever virus (RVFV), respectively. Diseases caused by members of these virus families do not only have a large public and animal health impact, but also an economic, socio-ethical and environmental impact. Given the increasing emergence of MBVs, surveillance is imperative to enable swift responses in terms of public and animal health policies, control measures and prevention strategies. Animals can be used to research and survey MBVs, either by observation of clinical cases or active surveillance such as serosurveys.

The overall objective of this thesis is to provide insight in the epidemiological situation, (response) surveillance options and potential risk of mosquito-borne viruses in animals in the Netherlands, in order to inform future research and surveillance strategies.

Very little is known about the current seroprevalence of MBVs in the Netherlands. Therefore, in **chapter 2**, a serosurvey among horses (*Equus caballus*) and dogs (*Canis lupus familiaris*) is described, species that are suggested to be good sentinels for orthoflavivirus circulation. Furthermore, I studied the knowledge and Health Belief (perceptions of health risks and factors influencing their health behaviour, including their knowledge, perceptions, and determinants of behavioural intention) of horse owners with regards to MBVs by using an online questionnaire. The serosurvey revealed a very low seroprevalence of WNV and Usutu virus (USUV) in both horses and dogs. The questionnaire revealed that Dutch horse owners have a high knowledge level and health belief with regards to MBVs compared to the Dutch general public. These results indicate that many dogs and horses remain susceptible to WNV and USUV, offering opportunities for trend analyses and surveillance. In **chapter 3**, the horses sampled in chapter 2 were tested for neutralizing SINV antibodies, together wild bird and human samples collected from bird ringers. In addition, mosquitoes and wild birds were tested for SINV RNA. One SINV positive bird and a seroprevalence of 0.82% (3/368) in horses was detected, while no humans or mosquitoes were (sero)positive. Thereafter, 10.91% (12/110) of wild bird samples were found seropositive in a 16 kilometre radius around the seropositive horses and the SINV positive bird. This study highlights the potential of using animal samples to detect local circulation of MBVs that would otherwise not be detected through human case detection.

Active sampling of animals for surveillance requires ethical permission as it is considered an animal experiment. Therefore, testing of existing (biobanked) samples may provide a more cost-effective solution and could lower animal discomfort. Therefore, in **chapter 4**, I tested 1,504 biobanked samples of Dutch wild boars (*Sus scrofa*) for USUV and WNV neutralizing antibodies. Then, I used a three-level Bayesian statistical model to analyse the neutralizing antibody responses. Serological interpretation of MBVs from the same serocomplex is challenging. Next to the unknown probability and extent of cross-reactivity, serological interpretation is complicated by an unknown time of infection and antibody persistence. In areas of co-circulation, interpretation of serological results is further complicated by the fact that individuals may be sequentially infected by multiple viruses from the same serocomplex. The statistical model revealed extensive cross-reactivity between USUV and WNV. The model results indicate that USUV prevalence was much higher in 2018 compared to the classical serological interpretation (four-fold neutralizing titre difference between viruses). Notably, the results show the presence of WNV infections in wild boars in or before 2018. These findings highlight the potential use of wild boars for orthoflavivirus surveillance, as these animals potentially have a higher exposure to vectors and samples are regularly collected for surveillance purposes of other pathogens.

Chickens are another species often used in MBV surveillance. Some studies suggest the use of sentinel chickens as indicators for human health risk. In **chapter 5**, repeated sampling of chickens in petting zoos and backyards was performed to investigate their potential use in MBV surveillance in the Netherlands. In total, 639 sera from 348 chickens were collected in three phases over the course of one year in two regions where WNV had been detected in 2020. Results of this study indicate circulation of both WNV and USUV in 2021, as chickens seroconverted during this period. This circulation of WNV went undetected by other surveillance methods such as syndromic surveillance in humans and horses. Furthermore, waning of USUV antibodies indicates that chickens could potentially be reused as sentinels in the following season(s). The use of petting zoo and backyard chickens thus seems a promising approach to detect orthoflavivirus circulation and potentially point towards public health risks.

It is essential to investigate the options for MBV (response) surveillance in animals to enhance preparedness for future outbreaks. Therefore, in **chapter 6**, I investigated the availability of sampling streams related to animals that are currently collected in the Netherlands. This resulted in an overview of ongoing sampling efforts in pets, livestock and wildlife. As many laboratories and institutes collect and test samples, the opportunities for rapid selection and sharing of these samples were investigated. This study revealed multiple barriers and requirements that need to be dealt with before samples can be shared between (research) institutions, hampering a rapid response to emergence of infectious pathogens.

As we live in a changing world, where drivers of disease emergence result in geographical spread and increase in circulation of MBVs, it is essential to investigate the effects of these changes on potential outbreak risk. In **chapter 7**, I developed a model to investigate the difference in potential outbreak risk of RVFV in the Netherlands between three scenarios. With this model I compare a reference (current) and two future scenarios representing a 'nature inclusive' and extensive (SSP1) and 'fossil-fuelled' highly intensive (SSP5) scenario around 2050. The outcomes reveal that the transmission season of RVFV is longest in the SSP5 scenario, and shortest in the reference scenario. The mean basic reproduction ratio (R_0) reaches the highest values in the SSP5 scenario, while a lower proportion of the country has an R_0 value above 1. More research is needed to investigate the sensitivity of the outcomes to parameter values and input data, especially with regards to the vector-to-host ratio. However, this study shows that the changes in temperature, vectors and livestock will have an effect on the outbreak risk of RVFV in the Netherlands in the future.

In **chapter 8** I interpret the findings of this thesis with regards to the current evidence of circulation of MBVs in the Netherlands, as well as describe potential approaches for future research and surveillance. Furthermore, I discuss the findings in the context of the One Health PACT consortium, which this project was a part of. Finally, I present an outlook with regards to emerging infectious diseases in a changing world and how humans have a key role to play in preparedness and prevention.

Samenvatting

Muggen-overdraagbare virussen vormen een opkomende bedreiging voor de diergezondheid en volksgezondheid in Nederland. Klimaatverandering, landgebruik, reizen en handel zijn aanjagers van deze opkomst. Zij zorgen ervoor dat het leefgebied van muggen en virussen wordt vergroot. Veel belangrijke muggen-overdraagbare virussen behoren tot de geslachten *Alphavirus, Orthoflavivirus* en *Phlebovirus*. Voorbeelden van virussen uit deze geslachten zijn respectievelijk het Sindbis virus (SINV), Westnijlvirus (WNV) en Riftdalkoortsvirus (RVFV). Ziekten die veroorzaakt worden door deze virussen hebben niet alleen een grote impact op de volksgezondheid en diergezondheid, maar uitbraken kunnen ook grote economische en maatschappelijke impact hebben, evenals impact op de (leef)omgeving en milieu. Aangezien muggen-overdraagbare virussen op steeds meer plekken voorkomen, is het van groot belang deze te monitoren. Deze monitoring zorgt ervoor dat men snel kan reageren met dier- en volksgezondheidsbeleid, controlemaatregelen en preventieve maatregelen om verdere uitbraken te voorkomen. Dieren kunnen gebruikt worden in deze monitoring van muggen-overdraagbare virussen. Enerzijds kunnen virussen die ziekte veroorzaken in dieren gemonitord worden door ziekte- en sterfgevallen van dieren in de gaten te houden. Daarnaast kan men ook actief infecties in dieren (die niet ziek zijn) monitoren door bijvoorbeeld het bloed van deze dieren te onderzoeken op virus of antistoffen. Op deze manier kan men erachter komen of deze dieren geïnfecteerd zijn (geweest), wat betekent dat het virus waarnaar gezocht wordt in de omgeving waar dit dier zich bevindt heeft gecirculeerd.

Het overkoepelende doel van dit proefschrift is om inzicht te verschaffen in de huidige epidemiologische situatie, opties voor (respons) monitoring en het potentiële risico van muggen-overdraagbare virussen in dieren in Nederland. Hiermee kunnen vervolgens toekomstige onderzoek en monitoring strategieën worden opgesteld. Dit is belangrijk om voorbereid te zijn en zo snel mogelijk te kunnen reageren op toekomstige uitbraken.

Er is zeer weinig bekend over de huidige seroprevalentie van muggen-overdraagbare virussen in Nederland. De seroprevalentie is het percentage individuen, in dit geval dieren, in een populatie dat positief test op (antistoffen tegen) een ziekteverwekker in een bloedmonster. De seroprevalentie kan dus worden onderzocht door bloedmonsters van een groep dieren te testen op antistoffen. **Hoofdstuk 2** van dit proefschrift beschrijft een seroprevalentie onderzoek bij honden en paarden. In de literatuur worden honden en paarden namelijk als potentieel goede verklikkers voor orthoflavivirus (zoals WNV) circulatie genoemd. Naast de testen in honden en paarden heb ik ook een online vragenlijst uitgezet bij paardeneigenaren en verzorgers. Deze vragenlijst was bedoeld om de kennis en “Health Belief” (dit zijn de percepties met betrekking tot gezondheidsrisico’s en factoren die hun gedrag hierin beïnvloeden) van

deze mensen te testen. De seroprevalentie studie liet zien dat er maar een heel klein percentage honden en paarden antistoffen had tegen WNV en Usutu virus (USUV, een ander orthoflavivirus). De vragenlijst liet zien dat Nederlandse paardeneigenaren zeer hoog scoren op kennis en Health Belief in vergelijking met de algemene Nederlandse bevolking (die in een andere studie was onderzocht). De seroprevalentie resultaten geven aan dat veel honden en paarden in Nederland nog geïnfecteerd kunnen raken met WNV en/of USUV, wat mogelijkheden biedt voor trendanalyses en monitoring op de langere termijn. In **hoofdstuk 3** hebben we de paardenmonsters verzameld in hoofdstuk 2 onderzocht op antistoffen tegen Sindbis virus samen met monsters van wilde vogels en mensen. Aanvullend hebben we ook muggen en wilde vogel monsters onderzocht op het virus zelf. We vonden Sindbis virus in één vogel, naast een seroprevalentie van 0.82% (3/368) in paarden. Geen van de mensen had antistoffen en ook vonden we het virus niet in muggen. Daarna bleek 10.91% (12/110) van de wilde vogels, die bemonsterd waren in een straal van 16 kilometer rond de positieve paarden en virus positieve vogel, antistoffen te hebben. Deze studie geeft aan dat diermonsters goed gebruikt kunnen worden om lokale circulatie van muggen-overdraagbare virussen te detecteren die niet zouden worden gevonden door alleen te kijken naar humane gevallen (ziekte).

Voor bemonstering van dieren in het kader van virus monitoring is ethische toestemming nodig, omdat dit wordt gezien als een dierproef. Daarom kan het testen van reeds verzamelde monsters (vanuit bijvoorbeeld een biobank) mogelijk een meer kosteneffectieve oplossing bieden, waarbij ook het ongemak voor dieren wordt verlaagd omdat er geen dieren specifiek voor de monitoring hoeven worden bemonsterd. In Nederland worden jaarlijks monsters verzameld en opgeslagen van wilde zwijnen om te testen op varkenspest en andere ziekten. Daarom heb ik in **hoofdstuk 4** 1504 monsters van Nederlandse wilde zwijnen getest op antistoffen tegen WNV en USUV. Daarna heb ik een statistisch model gebruikt om de resultaten te analyseren. Interpretatie van de hoeveelheid antistoffen tegen muggen-overdraagbare virussen is namelijk ingewikkeld. Dit komt omdat antistoffen van virussen uit eenzelfde familie soms zo op elkaar lijken dat ze met elkaar kruis-reageren. Dit betekent dat USUV antistoffen ook reageren in een WNV test, en het dus moeilijk maken om duidelijk te krijgen met welk virus het dier geïnfecteerd is geweest. Daarnaast weten we ook niet wanneer een dier precies geïnfecteerd is geraakt, en weten we ook weinig over hoelang antistoffen in het lichaam aanwezig blijven. In regio's waar meerdere soortgelijke virussen circuleren wordt dit nog verder gecompliceerd omdat dieren gedurende hun leven ook met twee (of meer) verschillende virussen geïnfecteerd geweest kunnen zijn geraakt. Het statistische model in hoofdstuk 4 liet, zoals verwacht, zien dat er veel kruisreactie was tussen WNV en USUV in de zwijnen monsters. De resultaten laten daarnaast zien dat de USUV seroprevalentie veel hoger was in 2018 dan wanneer de

uitslagen met de klassieke methode zouden worden geïnterpreteerd (die kijkt naar een 4-voudig verschil tussen de USUV en WNV uitslag). Een interessante bevinding was de aanwezigheid van WNV infecties in wilde zwijnen in, of voor, 2018. Deze bevindingen laten de mogelijkheden voor monitoring van orthoflavivirussen in wilde zwijnen zien. Wilde zwijnen hebben mogelijk een hogere kans gebeten te worden door muggen gezien hun leefomgeving. Daarnaast worden monsters van wilde zwijnen jaarlijks verzameld en opgeslagen voor andere doeleinden en kunnen dus ook worden gebruikt voor onderzoek naar andere ziekteverwekkers.

Kippen worden ook vaak gebruikt in het monitoren van muggen-overdraagbare virussen. Sommige studies suggereren dat 'verklikker' kippen als indicator kunnen worden gebruikt voor risico's voor de volksgezondheid. In **hoofdstuk 5** hebben we kippen van kinderboerderijen en uit achtertuinen herhaaldelijk bemonsterd om te onderzoeken of deze dieren kunnen worden gebruikt in orthoflavivirus monitoring in Nederland. In de loop van één jaar hebben we in drie fases in totaal 639 bloedmonsters van 348 kippen verzameld in twee regio's waar WNV was gevonden in 2020. Resultaten van deze studie laten zien dat zowel WNV als USUV hebben gecirculeerd in beide regio's in 2021. Dit weten we omdat kippen die eerst negatief waren gedurende deze periode antistoffen hebben ontwikkeld tegen één of beide virussen. Deze circulatie van WNV bleef onopgemerkt in andere monitoringssystemen zoals het observeren van ziekte bij paarden en mensen. Daarnaast laat de studie zien dat USUV antistoffen in kippen zodanig snel afnemen dat kippen mogelijk opnieuw kunnen worden ingezet in monitoring in een volgend seizoen. Het gebruik van achtertuin en kinderboerderij kippen lijkt dus een goede mogelijkheid om virus circulatie te detecteren en mogelijk te wijzen op volksgezondheidsrisico's.

Het is essentieel om de opties voor respons monitoring van muggen-overdraagbare virussen in dieren te inventariseren om de paraatheid voor toekomstige uitbraken te vergroten. Daarom heb ik in **hoofdstuk 6** onderzocht of er reeds beschikbare monsterstromen van dieren zijn in Nederland. Dit resulteerde in een overzicht van doorlopende monsterstromen in huisdieren, landbouwhuisdieren en wilde dieren. Omdat er veel laboratoria en instituten betrokken zijn bij het verzamelen en testen van monsters, werden ook de mogelijkheden voor het snel selecteren en delen van deze monsters onderzocht. De studie liet zien dat er meerdere hindernissen en voorwaarden zijn waar rekening mee gehouden moet worden voordat monsters gedeeld kunnen worden tussen (onderzoeks)instellingen. Dit zorgt voor een vertraging in de gewenste snelle respons op uitbraken van opkomende ziekteverwekkers.

Omdat we leven in een snel veranderende wereld, waar aanjagers van ziekteverspreiding zorgen voor een verhoogde circulatie en verspreiding van muggen-overdraagbare virussen, moeten we ook kijken wat de effecten zijn van deze

veranderingen op potentiële uitbraken. In **hoofdstuk 7** heb ik daarom een model ontwikkeld om het verschil in potentiële uitbraak risico van RVFV in Nederland te vergelijken tussen drie scenario's. Met dit model vergelijk ik een referentie (de huidige situatie) met twee toekomstscenario's die een natuur inclusief en een hoog-intensief scenario rond 2050 vertegenwoordigen. De uitkomsten laten zien dat het transmissie seizoen van RVFV het langst is in het hoog-intensieve scenario, en het kortst in het referentie scenario. Het gemiddelde reproductiegetal (R_0) behaalt de hoogste waardes in het hoog-intensieve scenario, terwijl een lager percentage van het totale landoppervlak een R_0 waarde boven de 1 laat zien. Er is meer onderzoek nodig om de gevoeligheid van de uitkomsten voor een aantal model waardes en data te onderzoeken, vooral met betrekking tot de vector-gastheer (mug/dier) verhouding. Desondanks laat deze studie al wel zien dat de veranderingen in temperatuur, muggen en landbouwhuisdieren een effect zullen hebben op het uitbraak risico van RVFV in Nederland in de toekomst.

In **hoofdstuk 8** interpreteer ik de bevindingen van dit proefschrift met betrekking tot het huidige bewijs voor circulatie van muggen-overdraagbare virussen in Nederland, evenals de mogelijkheden voor toekomstig onderzoek en monitoring. Daarnaast plaats ik de bevindingen in context van het One Health PACT onderzoeksconsortium, waar dit project onderdeel van uit maakt. Ten slotte presenteer ik een visie met betrekking tot opkomende infectieuze (dier)ziekten in een veranderende wereld en hoe mensen een sleutelrol spelen bij paraatheid en preventie.

Acknowledgements

P	R	A	R	O	D	Y	A	Y	U	O	N	E	H	E	A	L	T	H	P	A	C	T	W	V
I	O	G	E	F	T	H	J	J	W	I	M	Q	A	L	G	F	M	A	R	T	P	J	E	D
Z	B	T	I	N	E	S	L	E	Q	B	T	Y	R	S	V	O	A	D	O	L	F	O	A	R
H	C	B	N	B	P	W	O	A	E	T	M	V	A	I	E	B	R	P	Y	R	A	Z	P	E
O	K	L	A	A	S	J	A	N	P	H	G	O	W	H	Q	U	I	R	I	N	E	L	M	Q
E	F	T	G	F	Y	T	V	E	W	I	U	N	C	X	L	G	O	S	Y	T	M	P	A	P
Z	P	Q	W	O	T	V	E	T	E	R	I	N	A	R	I	A	N	S	I	C	L	A	R	A
O	I	H	U	N	P	D	V	J	M	Z	C	E	N	H	R	P	N	Z	T	O	V	L	T	U
S	B	I	W	S	L	V	A	W	T	A	I	N	I	Q	I	N	N	O	M	Z	I	E	H	L
H	C	U	C	O	O	U	L	H	P	Z	M	F	M	G	S	W	T	P	V	I	S	Z	A	I
O	V	T	O	G	U	E	E	E	F	K	E	E	A	M	G	Z	A	B	T	V	Z	Y	I	N
W	T	R	R	V	I	M	W	A	B	E	D	L	L	A	R	M	I	N	E	A	I	L	S	E
R	C	E	A	H	E	A	P	T	A	E	U	I	S	R	P	Z	W	R	G	M	L	L	A	Q
N	A	N	N	E	R	R	G	H	I	S	A	C	L	J	V	J	G	B	B	Z	V	J	M	A
P	H	A	B	N	U	I	C	E	G	T	R	I	I	O	B	O	R	I	S	V	I	L	G	N
Q	P	T	N	G	O	K	L	R	V	P	D	T	H	L	V	L	W	E	A	P	Z	M	N	N
B	R	E	G	T	J	E	A	P	M	U	O	Y	V	I	T	I	U	N	M	M	G	T	E	A
G	V	V	B	Z	I	N	Z	L	A	U	R	A	I	J	A	E	H	E	C	A	Y	A	T	B
I	P	T	A	M	A	Z	I	V	G	J	R	P	M	N	T	N	M	K	O	A	V	V	T	E
Y	M	A	N	N	E	K	E	P	S	O	P	H	I	E	P	N	R	E	G	R	U	M	Y	L
O	H	J	O	O	S	T	N	H	P	S	T	M	C	H	I	A	R	A	L	T	H	I	P	L
U	Z	G	U	H	V	M	G	H	I	E	A	T	H	G	U	W	T	G	V	E	L	J	O	E
V	P	T	K	C	A	T	H	E	L	I	J	N	E	L	V	G	Z	H	A	N	T	V	B	G
Z	M	A	R	I	A	N	N	E	U	Z	G	O	L	I	O	R	A	W	M	L	O	A	M	T
H	L	R	S	V	D	E	N	A	T	T	E	S	C	H	N	E	E	H	T	M	I	N	N	E

P	R	A	R	O	D	Y	A	Y	U	O	N	E	H	E	A	L	T	H	P	A	C	T	W	V
I	O	G	E	F	T	H	J	J	W	I	M	Q	A	L	G	F	M	A	R	T	P	J	E	D
Z	B	T	I	N	E	S	L	E	Q	B	T	Y	R	S	V	O	A	D	O	L	F	O	A	R
H	C	B	N	B	P	W	O	A	E	T	M	V	A	I	E	B	R	P	Y	R	A	Z	P	E
O	K	L	A	A	S	J	A	N	P	H	G	O	W	H	Q	U	I	R	I	N	E	L	M	Q
E	F	T	G	F	Y	T	V	E	W	I	U	N	C	X	L	G	O	S	Y	T	M	P	A	P
Z	P	Q	W	O	T	V	E	T	E	R	I	N	A	R	I	A	N	S	I	C	L	A	R	A
O	I	H	U	N	P	D	V	J	M	Z	C	E	N	H	R	P	N	Z	T	O	V	L	T	U
S	B	I	W	S	L	V	A	W	T	A	I	N	I	Q	I	N	N	O	M	Z	I	E	H	L
H	C	U	C	O	O	U	L	H	P	Z	M	F	M	G	S	W	T	P	V	I	S	Z	A	I
O	V	T	O	G	U	E	E	E	F	K	E	E	A	M	G	Z	A	B	T	V	Z	Y	I	N
W	T	R	R	V	I	M	W	A	B	E	D	L	L	A	R	M	I	N	E	A	I	L	S	E
R	C	E	A	H	E	A	P	T	A	E	U	I	S	R	P	Z	W	R	G	M	L	L	A	Q
N	A	N	N	E	R	R	G	H	I	S	A	C	L	J	V	J	G	B	B	Z	V	J	M	A
P	H	A	B	N	U	I	C	E	G	T	R	I	I	O	B	O	R	I	S	V	I	L	G	N
Q	P	T	N	G	O	K	L	R	V	P	D	T	H	L	V	L	W	E	A	P	Z	M	N	N
B	R	E	G	T	J	E	A	P	M	U	O	Y	V	I	T	I	U	N	M	M	G	T	E	A
G	V	V	B	Z	I	N	Z	L	A	U	R	A	I	J	A	E	H	E	C	A	Y	A	T	B
I	P	T	A	M	A	Z	I	V	G	J	R	P	M	N	T	N	M	K	O	A	V	V	T	E
Y	M	A	N	N	E	K	E	P	S	O	P	H	I	E	P	N	R	E	G	R	U	M	Y	L
O	H	J	O	O	S	T	N	H	P	S	T	M	C	H	I	A	R	A	L	T	H	I	P	L
U	Z	G	U	H	V	M	G	H	I	E	A	T	H	G	U	W	T	G	V	E	L	J	O	E
V	P	T	K	C	A	T	H	E	L	I	J	N	E	L	V	G	Z	H	A	N	T	V	B	G
Z	M	A	R	I	A	N	N	E	U	Z	G	O	L	I	O	R	A	W	M	L	O	A	M	T
H	L	R	S	V	D	E	N	A	T	T	E	S	C	H	N	E	E	H	T	M	I	N	N	E

Adolfo	Bregtje	Els	Joe	Marianne	Nnomzie	Sophie
Afonso	Cathelijne	Eva	Jolien	Mariken	Onehealthpact	SVdenatteschnee
Animals	Chiara	Felicity	Joost	Marion	Pauline	Szilvi
Annabelle	Clara	Heather	Jose	Marjolijn	Quirine	Thirza
Anneke	Clazien	Hoezoshow	Kees	Mart	Reina	Veterinarians
Anouk	Cora	Ilse	Klaas-jan	Martha	Renate	Wim
Armin	CUCo	Ines	Laura	Michel	Rob	You
Ayat	Dré	Iris	Liora	Minne	Rody	Yvonne
Bieneke	Eduardo	Jeanet	Louie	Nanne	Sam	Ziva
Boris	Eefke	Jo	Maarten	Netty	Sam	

Biography

Kiki Streng was born on the 19th of August, 1992 in Gouda, the Netherlands. Since she was four years old, she knew that she wanted to become a veterinarian. She finished the MSc Equine Health Care at Utrecht University in 2017. During her Master programme, she conducted a six month internship at the World Organisation for Animal Health (WOAH) in Paris, where she wrote her master thesis combined with a minor in Governance & Policy. Here, her interest for infectious diseases and (international) policy was spiked.

After graduation, she worked as a veterinarian for horses and small animals for three years. As her interest for infectious diseases and (international) animal health never disappeared during her time in practice, she applied for a PhD position which was brought to her attention by one of her master thesis supervisors. The position started on the 1st of April 2020, just after the first Dutch COVID-19 lockdown was announced. The project was part of the One Health PACT (Predicting Arbovirus Climate Tipping points) consortium, which comprised a collaboration between eight Dutch universities and other partners. Within this consortium, 26 PhD students and one Post-Doc performed research on arbovirus related topics withing a wide range of research fields.

Besides her work in veterinary practice and research, Kiki is very passionate about supporting animal welfare and veterinary professionals. This is why she was a board member (2018-2020) and president (2021) of the Young Veterinarians Platform which is part of the Dutch Royal Veterinary Association (KNMVD). In 2023 she joined the Equine Welfare Committee of the KNMVD as well as the Junior Network of the Dutch Council on Animal Affairs (Jong RDA).

Currently, Kiki is working parttime as a veterinarian in a small animal clinic in Zoetermeer.

Publications

Published

ORCID-ID: 0000-0001-6153-1428

Dellar, M., Streng, K., Van Bodegom, P., & Ibáñez-Justicia, A. (2024). Current and Future Probability of Occurrence of the Floodwater Mosquito *Aedes vexans* (Meigen, 1830) in the Netherlands. *Journal of Applied Entomology*, jen.13371. https://doi.org/10.1111/jen.13371

Streng, K.*, Atama, N.*, Chandler, F., Blom, R., van der Jeugd, H., Schrama, M., Koopmans, M. P. G., van der Poel, W. H. M., & Sikkema, R. S. (2024). Sentinel chicken surveillance reveals previously undetected circulation of West Nile virus in the Netherlands. *Emerging microbes & infections*, *13*(1), 2406278.
https://doi.org/10.1080/22221751.2024.2406278

Streng, K., Hakze-van der Honing, R. W., Graham, H., van Oort, S., de Best, P. A., Abourashed, A., & van der Poel, W. H. M. (2024). Orthoflavivirus surveillance in the Netherlands: Insights from a serosurvey in horses & dogs and a questionnaire among horse owners. *Zoonoses and public health*, 10.1111/zph.13171.

de Freitas Costa, E., Streng, K., Avelino de Souza Santos, M., & Counotte, M. J. (2024). The effect of temperature on the boundary conditions of West Nile virus circulation in Europe. *PLoS neglected tropical diseases*, *18*(5), e0012162.
https://doi.org/10.1371/journal.pntd.0012162

Streng, K.*, de Best, P. A.*, Timen, A., Koopmans, M. P. G., van der Poel, W. H. M., & Sikkema, R. S. (2023). Rapid response screening for emerging zoonotic pathogens, barriers and opportunities: A study for enhanced preparedness of the Netherlands. *One health*, *16*, 100507.
https://doi.org/10.1016/j.onehlt.2023.100507

P. de Best*, M. de Wit*, K. Streng*, M. Dellar, M. Koopmans (2021). Emerging Arboviral Diseases. *Nederlands Tijdschrift voor Medische Microbiologie* 29(3): 122-127.

Submitted

Streng, K.*, Holicki, C. M.*, Hesson, J. C., Graham, H., Chandler, F., Krol, L., Blom, R., Munger, E., van der Linden, A., Koenraadt, C. J. M., Schrama, M. J. J., De Bellegarde De Saint Lary, C., Visser, L. G., Oude Munnink, B. B., Lundkvist, Å., Koopmans, M. P. G., van der Jeugd, H., van der Poel, W. H. M., & Sikkema, R. S.. Sindbis virus (SINV) in the Netherlands: Evidence for local circulation in wild birds and horses.

Streng, K., Kampfraath, A.A., De Bruijn, N.D., Hakze-van der Honing, R.W., Bossers, A., Westendorp, S.T., Harders, F., Lecollinet, S., Bouwstra, R.J., van der Poel, W.H.M.. Usutu virus outbreak in a group of captive owls, the Netherlands.

* *Equal contribution*

Training and Supervision Plan (TSP)

The Basic Package (2.9 credits)	
WIAS Introduction Day (mandatory)	2020
Introduction course on Personal Effectiveness (recommended)	2020
WGS Scientific Integrity course (mandatory)	2021
WGS Ethics in Animal Sciences course (mandatory)	2021
Disciplinary Competences (19.9 credits)	
WIAS Research Proposal Writing	2020
MSc course: QVE-30806 MIDA (WUR)	2020
Introduction to Data Science with R and R Studio (WUR)	2021
Hands-on Veterinary Epidemiology (Utrecht University)	2022
Artikel 9 certification - Laboratory animal science course (Utrecht University)	2021
Basic Statistics (WUR)	2022
Reviewing 2 WIAS PhD proposals	2023
Professional Competences (7.2 credits)	
Research Data Management (WUR)	2021
The Essentials of Scientific Writing and Presenting (WUR)	2021
Workshop on Writing Propositions for your PhD (WUR)	2021
Scientific Publishing (WUR)	2021
WGS PhD Workshop Carousel (online)	2021
Better Waive than Worry' project organisation and grant writing (CuCo)	2022-2023
Scientific Writing (WUR)	2023
InterVision group (supervised by professional coach) (Dactari)	2020-2023
WIAS course The Final Touch: Writing the General Introduction and Discussion	2023
Societal Relevance (3.5 credits)	
Hoe?Zo! Theatershows voor kinderen - Outreach programma NWA wetenschapscommunicatie (NWA)	2021-2022
Course: Societal impact of your research (WUR)	2022
Media training (One Health PACT consortium), Rotterdam, the Netherlands	2022
Presentation Skills (4 credits)	
International Conference on Animal Health Surveillance (ICAHS), Copenhagen, Denmark, (poster)	2022
Dutch Arbovirus Research Network meeting (DARN), Leiden, the Netherlands, (oral)	2022
Society for Veterinary Epidemiology and Preventive Medicine (SVEPM), Toulouse, France (poster)	2023
WIAS annual conference (WAC), Wageningen, the Netherlands (poster)	2023
Symposium Dutch Society for Veterinary Epidemiology and Economics (VEEC), Deventer, the Netherlands (oral)	2023
Teaching competences (2 credits)	
Supervising MSc Internship student	2021
Supervising BSc student	2021
Total (minimum 30 credits)* **39.5**	

The research described in this thesis was financially supported by the project ‘Preparing for vector-borne virus outbreaks in a changing world: a One Health Approach’ (NWA.1160.1S.210), which is (partly) financed by the Dutch Research Council (NWO).

Thesis cover by Jolien Streng

Printed by ProefschriftMaken, de Bilt, the Netherlands