

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

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study presents a significant and timely analysis of Usutu virus (USUV) circulation in the Netherlands over a seven-year period, with additional insights into West Nile virus (WNV). The work is particularly relevant given the increasing threats posed by arboviruses in Europe due to climate change, land-use changes, and globalization. The robust sampling framework—incorporating live bird surveillance, citizen-science data, zoo referrals, and mosquito trapping—demonstrates a comprehensive approach to USUV epidemiology and highlights the value of wildlife surveillance as an early warning system for emerging zoonotic threats, including WNV. The study's strengths lie in its seven-year sample collection, which includes serological and genomic characterization of USUV lineages (Africa 3 and Europe 3), as well as its role in detecting a human WNV outbreak. However, while the descriptive analysis is thorough, the study does not fully explore the ecological and epidemiological factors that may explain USUV's predominance over WNV, which would enhance its scientific impact.

Major Comments

The paper is well written; however, the research question is not clearly articulated. What specific hypotheses drive this study? While the motivation is evident, the paper reads more like an observational study rather than a research paper with a well-defined question. Consider changing the title to properly reflect the observational aspect of the study.

The study demonstrates that USUV is more prevalent in bird populations than WNV, yet the underlying reasons for this pattern remains unexplored. The discussion should address the potential ecological or epidemiological mechanisms driving this trend. For instance, while the study provides a valuable characterization of bird species susceptible to USUV and WNV infections in the Netherlands, only seven birds were found to be infected with WNV. The authors do not propose any hypotheses to explain this disparity. Are there existing studies that explore the role of bird host immunity on susceptibility to USUV and WNV? Additionally, could mosquito coinfections influence transmission dynamics, potentially leading to a higher likelihood of USUV transmission over WNV? Including relevant references and expanding on these possible factors would enhance the study's scientific impact and provide a more comprehensive interpretation of the findings.

Although the bird sampling effort is impressive, the presentation of results may unintentionally exaggerate the prevalence of positive detections. For example, the circle sizes in Figure 2 and the raw counts in Figures 3A and 4 may create a misleading impression. Consider adjusting the visual representation to ensure a more proportional interpretation of the data.

Minor Comments

L22: No need to specify Homo sapiens.

L63: "The highest number of deaths associated with USUV infection (n=208)"—208 out of how many? Is this the same 4,415 reported earlier? Clarify.

L69: 69: The phrase states, "WNV was detected in 7 live landbirds"; however, Supplementary Table S2 indicates that 8 landbirds tested positive for WNV RNA. Could you please clarify this discrepancy?

L74-75: Numbers should be accompanied by the total number of tested individuals.

Figure 1: The use of circles to represent sample sizes per year may not be the best choice. The bar format used in Figure 3 would likely improve readability and clarity.

Figure 2: While visually appealing, the current representation may be misleading given the number of positive samples. A pie chart within the circles, rather than a nested-circle approach, could better depict proportions. Consider revising.

L318-325: This section does not describe a spatial analysis but rather an aggregation of data.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Thank you for inviting me to review the manuscript 'An integrated approach uncovers the emergence and dynamics of Usutu and West Nile viruses in the Netherlands' by Münger et al. From the off I would like to commend the authors on the effort required to generate the presented data, which covers significant surveillance activities across the Netherlands between 2016-2022, inclusive. The combination of active and passive surveillance, incorporating both professionals and citizen scientists, provides a detailed, One Health approach, that acts as a model for how surveillance should be undertaken for emerging and endemic arboviruses, well done! The choice of statistical analyses, and execution of figures are well thought out and engaging. However, in general I found the manuscript to be lacking a definitive synthesis of the findings over seven years, and to my mind requires some additional thought. Below I have detailed some broader major recommendations, and some minor comments that I believe should be addressed.

Major point 1. The work captures, describes and presents surveillance data, in a descriptive manner, which indeed elucidates some of the wider ecology of these emerging arboviruses, especially in avian hosts. However, no real thought is given into drivers of outbreaks, drivers of establishment, drivers of expansion or peak enzootic transmission in spring 2019 and summer 2022. The authors detailed surveillance data (perhaps combined with other publicly available data sets) could help to at least speculate on such drivers of arboviral epidemiology in temperate areas, and would make for a more compelling read that improves our understanding more generally.

Major point 2. Key results presented in this study have already been published. While this is not necessarily an issue (if steps are taken to further understanding of arboviral epidemiology through their synthesis), repeating findings in a descriptive manner without formulating a narrative on wider implications for emerging arboviruses, weakens the justification for their inclusion and by extension weakens the manuscript.

Minor points

Abstract: You state that USUV was first detected in 2016, please specify this detection was in the Netherlands

Throughout ms: Where date ranges, month ranges and year ranges are provided, could the authors confirm if the event occurred between those dates, or inclusive of those dates. E.g. 'occurring between July and November, inclusive'. Would indicate that July and November were included in the sampling, which I expect is accurate of what happened and is useful in trying to understand the temporal dynamics of Orthoflaviviruses.

L2: The authors mention reservoir hosts throughout the manuscript, but don't mention amplifying hosts, or indicate which species may be involved as amplifying and /or reservoir hosts for Orthoflaviviruses more generally from their surveillance findings.

L6: The authors say 'in recent years', could you be more specific please as this is quite subjective, and your description appears to capture c. 30 years

L6-8: Termed natural expansion? I believe it would be important to capture this to contextualise that these viruses do naturally expand and expansion is not solely driven by human activity.

L8: Regions in Europe with established Aedes species? If so please specify, as this could indicate areas outside Europe with invasive mosquito species.

L16: Use of 'their' for viruses to my mind is incorrect as it implies belonging, please edit to read as 'Culex pipiens mosquitoes are the main vector'

L18: The authors mention these species have been particularly affected, to my mind this doesn't capture the true impact. For blackbird especially, USUV has been linked to disease mediated population declines. There are a number of appropriate references that show specific population declines of blackbird in areas where USUV has emerged or is circulating across Europe.

L25-26: 1% neurological symptoms – 1% of total infections (including asymptomatic), or 1% of the 20% that develop febrile illness? Please be specific.

L29: This statement implies that USUV spread from Austria, was Austria the first incursion (detected or not) of USUV? It is likely that other outbreaks had occurred previously, and these also contributed to geographic expansion across Europe. Could the authors rephrase this statement accordingly please.

L32: The authors claim that 'large' outbreaks occurred in humans and animals. This is quite a subjective statement, could the authors provide some context please, i.e. number of confirmed infections etc.

L40: I disagree that circulation of one virus could influence the other. More appropriate to say that presence of one, may be an indicator that conditions are suitable for other arboviruses that have a similar ecological niche.

L46: 'Well before human or veterinary disease', please remove 'well', this is a subjective term that could be misinterpreted. I do agree however that detailed wildlife surveillance can detect emergence/outbreaks of arboviruses prior to human

circulation.

Figure 2: Is fantastic, well done, what an intuitive way to present the data.

L95-97: The authors say 'initially', I assume they mean in 2016, but could they please be specific when describing temporal data, as it is key for following the outbreak and understanding epidemiology. In addition, they give some region details, but perhaps some wider information on specific habitats could be useful – urban, rural, near wetlands etc and how that might link to their findings.

L103-104: I would specify that this is RNA detection (I assume it is) and not just seroconversion as RNA implies either transmission is still occurring, or that viraemia in these hosts lasts longer than expected. Might be worth mentioning in main ms which host species and sample types these were.

L105-106: Why were these species most frequently tested, a sampling bias due to mortality, for e.g., or targeted surveillance for key host species?

L108: 'peaking at 26.7% in Eurasian blackbirds and song thrushes' Could the authors confirm is 26.7% is the combined peak or both species had a similar individual peak? If the former (which I assume it is), could the authors further break that peak down into blackbird and song thrush specific values in the main ms as this would be helpful for understanding exposure in thrushes (one species highly susceptible, another not).

L109: 'In 2016, serum testing was very limited' Could the authors explain why and quantify this statement.

L109-111: The seroprevalence data seems to mirror your molecular detections, might be worth explicitly saying that in the ms.

L111-112: Why did serum sampling remain scarce?

L113: You use PMA abbreviation here and I don't believe I have seen it in the ms in full, only later in the methodology.

Figure 4 and 5: Really lovely, informative panels, great!

L155: Over the last how many decades?

L158: You have talked about animal health previously, so this should read as, 'public and animal health and environmental strategies'.

L167-168: This statement implies USUV infection in any bird often led to death, which is inaccurate. Primarily blackbird, with notable mortality recorded in some other groups, great grey owls, for e.g., could the authors amend this statement accordingly please.

L246-247: Is WNV considered established in the Netherlands? I would tone this statement down, good evidence for USUV, less so for WNV. Also, the word phenomenon seems misplaced here. These arboviruses are emerging and persisting in temperate areas across Europe, so not really a phenomenon.

Discussion in general: As per my major comment, the discussion is quite descriptive, without any major steps forward in our understanding. The authors have a unique and valuable dataset involving seven years of surveillance activity and I believe they could make more of their assumptions on arboviral epidemiology, or risk modelling.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

We have read the responses to our comments and to the other reviewers. We think they did a good job addressing the main points. They clarified the study's main research questions and hypotheses and explained more clearly that this is an observational study. They also improved the discussion by adding more explanation about why USUV is more common than WNV in the Netherlands, with relevant literature and possible mechanisms.

Regarding the figures, they added scales, annotations, and made sure readers can find the raw data, so the visual presentation is clearer. We also see that they responded well to the other reviewer's comments — they added more context about environmental drivers and how this surveillance data could help future modelling. They also clarified their use of terms like reservoir and amplifying hosts, and fixed some details and phrasing to be more precise.

Overall, we think the revised manuscript is clearer, more complete, and stronger. We don't have any major concerns left.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

I would like to thank the authors for their comprehensive response to my review. Following revision of the manuscript, I have no further comments to make, and would like to say well done to all those involved in its completion. The manuscript makes for an engaging read and improves our understanding of arbovirus epidemiology in Europe.

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

NCOMMS-25-11031

Response to Reviewers

We sincerely thank the three reviewers for their thoughtful, constructive, and insightful comments. These have helped us improve the quality and clarity of the manuscript. Below, we provide responses to each comment and indicate how we have made modifications in the manuscript.

REVIEWER #1

This study presents a significant and timely analysis of Usutu virus (USUV) circulation in the Netherlands over a seven-year period, with additional insights into West Nile virus (WNV). The work is particularly relevant given the increasing threats posed by arboviruses in Europe due to climate change, land-use changes, and globalization. The robust sampling framework—incorporating live bird surveillance, citizen-science data, zoo referrals, and mosquito trapping—demonstrates a comprehensive approach to USUV epidemiology and highlights the value of wildlife surveillance as an early warning system for emerging zoonotic threats, including WNV. The study's strengths lie in its seven-year sample collection, which includes serological and genomic characterization of USUV lineages (Africa 3 and Europe 3), as well as its role in detecting a human WNV outbreak. However, while the descriptive analysis is thorough, the study does not fully explore the ecological and epidemiological factors that may explain USUV's predominance over WNV, which would enhance its scientific impact.

Major Comments

1. The paper is well written; however, the research question is not clearly articulated. What specific hypotheses drive this study? While the motivation is evident, the paper reads more like an observational study rather than a research paper with a well-defined question. Consider changing the title to properly reflect the observational aspect of the study.

While the study is observational, its hypotheses can be explicitly framed. We have modified the last paragraphs of the introduction to read as follows:

"Here, we present the results of seven years of research (2016–2022, inclusive) on mosquito-borne viruses in birds and mosquitoes across the Netherlands. In light of the expanding spread of mosquito-borne viruses in Europe, we hypothesized that, despite earlier assumptions that climatic conditions would limit the establishment of mosquito-borne diseases, the Netherlands—with its water-dominated ecosystems, diverse avifauna, and major transport hubs—might be vulnerable to mosquito-borne disease outbreaks. This was further supported by observed large scale outbreaks among animals of Schmallenberg virus and bluetongue virus, both vector-transmitted viral infections (Hoffmann et al., 2012; Beer et al., 2013). Our primary objective was to detect the circulation of emerging mosquito-borne viruses in the Netherlands. Once emerging mosquito-borne viruses were detected, we aimed to identify which bird and mosquito species could become infected, to assess the potential for their local maintenance over time, to characterize patterns in their emergence and spread, and to gain insights into their epidemiology in this previously non-endemic region.

To address these questions, as well as to provide early warning for future outbreaks, we established a nationwide integrated sampling framework in a unique collaboration of ecologists, ornithologists, entomologists, veterinarians, virologists, and citizens. A multi-tiered sampling scheme was designed to collect samples from free-ranging live and dead birds, birds deceased in captivity, and mosquitoes, which were screened for mosquito-borne viruses using molecular and serological methods (Fig. 1). Using this integrated framework, we detected and characterized the emergence of USUV and WNV, documented their circulation in birds and mosquitoes, and identified patterns of virus persistence in the Netherlands. We thereby generated foundational data that could support future modelling, risk assessment, targeted surveillance, and outbreak prediction efforts."

Regarding the title, to comply with journal guidelines, we shortened it to: *"One Health approach uncovers emergence and dynamics of Usutu and West Nile viruses in the Netherlands"*. We believe this title reflects the observational nature of the study.

- The use of the term *"uncovers"* – meaning "to make known" or "to bring to light" – suggests discovery through systematic observation and does not imply experimental manipulation or hypothesis-driven causality testing.
 - The focus on *"emergence and dynamics"* is consistent with terminology commonly used in epidemiological studies, with the term *"dynamics"* used here to encompass temporal and spatial patterns over time as well as the host species involved in disease transmission, in a concise manner.
2. The study demonstrates that USUV is more prevalent in bird populations than WNV, yet the underlying reasons for this pattern remains unexplored. The discussion should address the potential ecological or epidemiological mechanisms driving this trend. For instance, while the study provides a valuable characterization of bird species susceptible to USUV and WNV infections in the Netherlands, only seven birds were found to be infected with WNV. The authors do not propose any hypotheses to explain this disparity. Are there existing studies that explore the role of bird host immunity on susceptibility to USUV and WNV? Additionally, could mosquito coinfections influence

transmission dynamics, potentially leading to a higher likelihood of USUV transmission over WNV? Including relevant references and expanding on these possible factors would enhance the study's scientific impact and provide a more comprehensive interpretation of the findings.

We have added a passage to the discussion addressing these different elements and included relevant references (L298 to 317), it reads:

"Molecular and serological analyses showed that WNV was less prevalent than USUV in bird populations, with molecular detections of WNV limited to 7 birds. Globally, WNV or WNV antibodies have been detected in over 100 bird species, many of which occur as free-ranging birds in Europe (Nikolay, 2015), and WNV circulates widely among European bird populations (Tolsá et al., 2018). The lower prevalence of WNV in the Netherlands likely reflects its later emergence and lower transmission levels compared to USUV. As discussed above, USUV likely circulated undetected in its early phase in the Netherlands or in neighbouring regions, prior to the initiation of the integrated arboviral monitoring in birds and mosquitoes described in this study. Likewise, WNV may currently be in a silent early phase of low-level circulation, detectable only through enhanced arboviral monitoring (Streng et al., 2024), and potentially on the cusp of causing a larger outbreak in the Netherlands. However, given their overlapping transmission cycles, host and vector species, and antigenic relatedness, widespread USUV circulation may not only suggest the potential for increased local circulation of WNV – interactions between the two viruses in regions where they co-circulate may also occur. This possibility is supported by experimental studies: prior WNV infection in wild House Sparrows from the United States conferred protection against secondary USUV infection (Bosco-Lauth et al., 2024), while prior USUV infection in Domestic Geese reduced viral load and disease severity upon secondary infection with WNV (Reemtsma et al., 2023). In *Cx. pipiens*, prior exposure to USUV via infectious blood meals significantly reduced subsequent WNV infection and transmission, although WNV outcompeted USUV when mosquitoes were simultaneously exposed to both viruses (Wang et al., 2020). In the Netherlands, the broad host range, wide geographical distribution, and established enzootic presence of USUV may have impeded WNV transmission and spread following its emergence."

3. Although the bird sampling effort is impressive, the presentation of results may unintentionally exaggerate the prevalence of positive detections. For example, the circle sizes in Figure 2 and the raw counts in Figures 3A and 4 may create a misleading impression. Consider adjusting the visual representation to ensure a more proportional interpretation of the data.

We appreciate the reviewer's concern. To mitigate the risk of misinterpretation, we have now added a scale to **Figure 2** and had annotated the exact prevalence values (and raw counts for dead birds). Detailed data on counts and prevalence for all tested species are fully provided in the supplementary materials as spreadsheet tables (**Supplementary Data 1**), allowing thorough and precise interpretation by readers. We have now explicitly mentioned this in the manuscript when referring to **Figure 2**. We would also like to highlight that all raw data have been made available, as described in the **Data availability** statement, ensuring transparency. Our reasoning for the current visualizations is outlined below.

In **Figure 2**, we chose proportional circles to help readers appreciate and compare detection rates across species. While pie charts were suggested in another comment of the reviewer, we found that these may not serve this purpose as effectively. Small pie segments are notoriously difficult to interpret and compare, and using pies would obscure the underlying sample sizes—an important factor influencing the likelihood of detection. We sought to visually facilitate exploration of subtle patterns across species for our readers.

In **Figure 3A**, we use epidemic curves –a widely used visualization in epidemiology– showing raw counts of positive detections, alongside raw counts of samples tested to contextualize detection trends. We fully agree that prevalence is a highly informative metric, and for the study that best allows reliable prevalence tracking over the seven-year monitoring period (i.e., the live, free-ranging landbirds), we additionally present this explicitly in **Figure 3B**. For dead birds, however, where mortality, likely due to disease (the condition for sampling and testing), is expected to be directly associated with infection, absolute numbers offer particularly meaningful insights. For this reason, we found raw counts to be more appropriate for this specific dataset. To clarify, we have moved the sentence "Reported dead free-ranging birds were selected for necropsy at the Dutch Wildlife Health Centre (DWHC) based on carcass freshness and suspected disease-related death." from the supplementary methods to the methods of the main Methods section (L394).

In **Figure 4**, raw counts were used for dead birds following the same reasoning as outlined above. For live birds, we also chose to display raw counts because our sampling effort was not uniform across sites. In this summarizing overview of a seven-year period, prevalence estimates would be influenced by variation in sampling intensity, the proportion of landbirds versus waterbirds at each location, and the timing of sampling at less intensively sampled sites. Additionally, we aimed to visualize the sampling effort alongside the detection of viruses and antibodies. A visualization of USUV seroprevalence at live bird ringing sites across the Netherlands over time is included in the supplementary materials (**Supplementary Fig. 3**).

Minor Comments

1. L22: No need to specify *Homo sapiens*.

Now at L24: We are aware that the standard taxonomic convention to provide the Latin name of a species at its first mention is often waived for humans. In the context of our study, which addresses a highly interspecific topic, we chose to specify the Latin name for humans, as we do for other species, as a subtle but deliberate reminder to readers that we humans are one

species amongst many, interacting within a shared environment. We can omit this specification if the reviewer feels strongly, but this was our rationale for including it.

2. L63: "The highest number of deaths associated with USUV infection (n=208)" –208 out of how many? Is this the same 4,415 reported earlier? Clarify.

This is out of the total of dead blackbirds tested. We have modified the sentence to read (now at L84): "Eurasian Blackbirds had the highest USUV prevalence among live birds (n=106/4'415, 2.4%) and the highest number of deaths associated with USUV infection (n=208/399)."

3. L69: The phrase states, "WNV was detected in 7 live landbirds"; however, Supplementary Table S2 indicates that 8 landbirds tested positive for WNV RNA. Could you please clarify this discrepancy?

Now at L91: The full sentence in question reads, "WNV was detected in 7 live landbirds (one of which tested positive twice, with a 5-day interval)." The number "8" in Table S2 refers to the total number of WNV RNA-positive detections, where two of these detections are from a same individual bird. As specified in the Methods section, "Recapture and resampling of ringed birds occurs frequently during the breeding season, and the numbers presented in this study reflect the number of sampling events of individual birds, rather than the absolute number of individual birds."

We chose not to exclude recapture events, as they provide valuable insight into the infection dynamics within live bird populations at each specific time point. Most recaptures occur over longer intervals, and repeated molecular positives are rare in our dataset. However, in the case of WNV, given the small number of detections, we thought it was important to specify that, by a luck of the draw, two of the detections were from a same individual.

4. L74-75: Numbers should be accompanied by the total number of tested individuals.

We have modified these lines to read (now at L96-99): "Out of the total of 22'700 live landbirds captured, a serum sample was collected from a subset of 4'176 live landbirds (18.4%). Among these 4'176, 240 tested positive for USUV-neutralizing antibodies (5.7%), 53 for WNV-neutralizing antibodies (1.3%) and 40 tested positive for both USUV- and WNV-neutralizing antibodies (1%), without the possibility to discriminate between the two."

5. Figure 1: The use of circles to represent sample sizes per year may not be the best choice. The bar format used in Figure 3 would likely improve readability and clarity.

To improve clarity and readability of **Figure 1**, we have directly annotated the exact sample numbers on the proportional circles in Figure 1, in lieu of using a scale. The purpose of this figure is to provide a simple overview of the different studies and their timelines, rather than to convey detailed quantitative data (detailed numbers of tested samples per month per study are presented in Figure 3 in bar format). We acknowledge that in the article format of Nature Communications—where the Methods are presented at the end—such an overview figure is more useful when placed earlier in the text. We have therefore moved this figure to the Introduction.

6. Figure 2: While visually appealing, the current representation may be misleading given the number of positive samples. A pie chart within the circles, rather than a nested-circle approach, could better depict proportions. Consider revising.

We have addressed this point under **Major Comment 3**.

7. L318-325: This section does not describe a spatial analysis but rather an aggregation of data.

We have renamed this section: "Spatial clustering and aggregation of sampling data"

REVIEWER #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER #3

Thank you for inviting me to review the manuscript 'An integrated approach uncovers the emergence and dynamics of Usutu and West Nile viruses in the Netherlands' by Münger et al. From the off I would like to commend the authors on the effort required to generate the presented data, which covers significant surveillance activities across the Netherlands between 2016-2022, inclusive. The combination of active and passive surveillance, incorporating both professionals and citizen scientists, provides a detailed, One Health approach, that acts as a model for how surveillance should be undertaken for emerging and endemic arboviruses, well done! The choice of statistical analyses, and execution of figures are well thought out and engaging. However, in general I found the manuscript to be lacking a definitive synthesis of the findings over seven years, and to my mind requires some additional thought. Below I have detailed some broader major recommendations, and some minor comments that I believe should be addressed.

Major Comments

1. The work captures, describes and presents surveillance data, in a descriptive manner, which indeed elucidates some of the wider ecology of these emerging arboviruses, especially in avian hosts. However, no real thought is given into drivers of outbreaks, drivers of establishment, drivers of expansion or peak enzootic transmission in spring 2019 and summer 2022. The authors detailed surveillance data (perhaps combined with other publicly available data sets) could help to at least speculate on such drivers of arboviral epidemiology in temperate areas, and would make for a more compelling read that improves our understanding more generally.

We have expanded the discussion of the potential ecological and environmental drivers of virus emergence and establishment (L324-367 in the revised manuscript). These new paragraphs synthesizes our own findings with publicly available climatic data and relevant literature. We discuss how climatic variables, avian host population dynamics, and host immunity may jointly influence arbovirus transmission. We also clarify that our study provides a baseline dataset that can support future hypothesis-driven modeling studies aimed at formally identifying the key drivers of arbovirus emergence, establishment, and expansion.

"The emergence and maintenance of USUV and WNV in the Netherlands and in other parts of Western Europe is likely driven by changes in environmental conditions, becoming favourable for arbovirus establishment in locally present (non-introduced) mosquito species, larger outbreaks in wild birds, and occasional zoonotic transmission. Climate change has recently been formally identified as a critical driver of increased WNV circulation risk in Europe (Erazo et al., 2024). Within this expanding zone of environmental suitability, both short-distance bird movements (e.g., foraging flights) and long-distance migration likely facilitate virus introduction and geographic spread (Mancuso et al., 2022; Lu et al., 2024; Van Irsel et al., 2025). The Netherlands has extensive wetland habitats, rich in mosquitoes and avian hosts, and, under favourable climatic conditions, appears to provide the key ecological parameters for arbovirus transmission cycles. Presence of wetlands in an area is identified as an important driver for a higher WNV disease incidence in animal and humans in Europe (Giesen et al., 2023).

Our findings suggest that the intensity of USUV and WNV activity is regulated by a dynamic interplay between climatic conditions, host population dynamics, and host immunity. Prior to the emergence of USUV and until the peak of the first transmission period, the Netherlands experienced five consecutive very warm years (2014-2018), consistent with a broader trend of a warming climate (Royal Netherlands Meteorological Institute (KNMI), 2019). Within our study period, 2018 and 2022 stood out as very warm and dry years, marked by extremely hot summers, which coincided with peaks in USUV transmission (Royal Netherlands Meteorological Institute (KNMI), 2019, 2023) (**Supplementary Fig.7**). Remarkably hot summers have similarly coincided with peaks in USUV transmission in Austria and Germany (Rubel et al., 2008; Bergmann et al., 2023). More generally, studies have linked the occurrence of USUV and WNV to high winter, spring, and summer temperatures as well as to precipitations in summer (Marcantonio et al., 2015; Cheng et al., 2018; Marini et al., 2021; Watts et al., 2021; Farooq et al., 2023). Within the Netherlands, regional variations in USUV occurrence were also associated with climatic conditions, particularly temperature in late winter, early spring, and summer (Van Irsel et al., 2025).

However, the decline in USUV detections observed in this study from 2019 onwards can *a priori* not be solely attributed to climatic factors: 2019 and 2020 were warm years with climatic conditions not markedly different to those of 2016 and 2017 (Royal Netherlands Meteorological Institute (KNMI), n.d.), years favourable for USUV emergence and spread. Instead, host-related factors likely played a key role in this decline. In 2019, Eurasian Blackbird populations across the Netherlands were estimated to have declined by 30% due to USUV-related mortality (Van Irsel et al., 2025), and we observed peak seroprevalence in this species in spring and summer. A subsequent recovery period in avian host population density and susceptibility may have been necessary before USUV transmission could intensify again, potentially prolonged by less favourable climatic conditions in 2021. A cyclic pattern in USUV infections – characterized by a few years of high-level circulation followed by several years of low activity – has also been observed in Austria (Weidinger et al., 2020). There, a large outbreak from 2001 to 2003 was associated with substantial bird mortality, followed by a 13-year period with few USUV-associated bird deaths before a resurgence (Chvala et al., 2007; Meister et al., 2008; Weidinger et al., 2020). An epidemic model developed for Austria supports this cyclic pattern, suggesting that rapid waning of herd immunity in bird populations permits recurring outbreaks (Rubel et al., 2008). In our study, we observed a rapid decline in seroprevalence among Eurasian Blackbirds following peak infection period, a pattern also described in sentinel chickens (Streng et al., 2024; De Bruin et al., 2025). Meanwhile, population indices for Eurasian Blackbirds showed signs of recovery from 2020 onwards (Van Irsel et al., 2025).

The baseline datasets generated in this study provide a valuable foundation for formal hypothesis testing and model development to analyse the dynamics of mosquito-borne viruses. Future research using ecological niche models,

transmission models, or phylodynamic analyses incorporating covariates could help identify key drivers of arbovirus circulation, establishment, and spread. This, in turn, could refine predictive outbreak models, inform targeted interventions, and support the identification of robust risk indicators.”

2. Key results presented in this study have already been published. While this is not necessarily an issue (if steps are taken to further understanding of arboviral epidemiology through their synthesis), repeating findings in a descriptive manner without formulating a narrative on wider implications for emerging arboviruses, weakens the justification for their inclusion and by extension weakens the manuscript.

The first detections of USUV and WNV in specific hosts and locations in the Netherlands have indeed been reported earlier in the context of early warning and outbreak reporting, which is considered to be a standard in public health relevant infectious disease research. We refer to those studies where relevant. However, those initial reports were only presenting a small part of our total findings, even for the first transmission seasons that these publications addressed.

This is the first study to comprehensively integrate seven years of arbovirus surveillance data across host species, sampling schemes, and virus types (USUV and WNV). Particular attention was given to releasing all raw data – including negative test results – which are often omitted in rapid-response publications but are critically important for future modelling studies, and risk assessment.

In response to this comment and Major Comment 1, we have also expanded the discussion to offer a deeper interpretation of the observed patterns and to explore potential ecological and epidemiological drivers of arbovirus dynamics.

Minor Comments

1. Abstract: You state that USUV was first detected in 2016, please specify this detection was in the Netherlands

We modified the passage to “USUV was first detected in the Netherlands in 2016, ...”

2. Throughout ms: Where date ranges, month ranges and year ranges are provided, could the authors confirm if the event occurred between those dates, or inclusive of those dates. E.g. ‘occurring between July and November, inclusive’. Would indicate that July and November were included in the sampling, which I expect is accurate of what happened and is useful in trying to understand the temporal dynamics of Orthoflaviviruses.

The date ranges provided were indeed inclusive. To clarify, we have added the term “inclusive” after any date range or reformulated the text to use more precise dating.

3. L2: The authors mention reservoir hosts throughout the manuscript, but don’t mention amplifying hosts, or indicate which species may be involved as amplifying and /or reservoir hosts for Orthoflaviviruses more generally from their surveillance findings.

We thank the reviewer for bringing our attention to this point. The terminology surrounding hosts in arbovirus ecology is complex, with multiple and sometimes contradictory definitions for specific terms (see for example Kuno G, et al., “Vertebrate Reservoirs of Arboviruses: Myth, Synonym of Amplifier, or Reality?” 2017; “Identifying Reservoirs of Infection: A Conceptual and Practical Challenge.” 2002 or Ashford RW. “When is a reservoir not a reservoir?” 2003). To clarify, we have used specific terms as follows:

L2: “These viruses are maintained in infection cycles involving mosquitoes as vectors, and vertebrates as amplifying hosts.”

L16: “Various bird species contribute to the amplification and maintenance of these viruses, with some species showing high susceptibility to disease.”

Then, we have extended the discussion on avian host and specified the framework for the terminology we used. It now reads as follows (L215-248):

“Combining molecular and serological testing in live birds with molecular testing in dead birds allowed us to comprehensively cover bird species involved in the ecology of USUV in the Netherlands. The strongest evidence of infection and associated mortality was observed in Eurasian Blackbirds, and high numbers of detections were noted in Great Grey Owls deceased in captivity, consistent with observations from other countries (Nikolay, 2015; Clé et al., 2019). Our findings also add to the growing evidence of infection and exposure in a broader range of bird species, including members of the Corvidae, Columbidae, and raptors families (Accipitridae and Strigidae). Hereafter, we use the term *reservoir system* to refer to the ecological network in which the virus is maintained indefinitely, in the context of Western Europe (Ashford, 1997). Several hosts (including intermediate hosts or vectors) likely collectively constitute the reservoir system. We call vertebrate hosts that form an essential part of this system *reservoir hosts*.”

While USUV caused mass mortality in Eurasian Blackbirds, the frequent detection of USUV and USUV-neutralizing antibodies in living individuals without visible symptoms suggests that this species can also carry the virus while remaining fit or surviving mild illness. Eurasian Blackbirds are the most common breeding birds in the Netherlands, and research in the USUV- and WNV- affected area of Northern Italy has shown a feeding preference of *Cx. pipiens* for this species (Rizzoli et al., 2015). Eurasian Blackbirds are therefore likely reservoir hosts, playing a key role in sustaining viral transmission despite the high

mortality observed. This species was also the most frequently found USUV RNA-positive during winter months. While RNA detection alone does not confirm active infection or the presence of viable virus, these findings raise the possibility that Eurasian Blackbirds may contribute to USUV persistence across seasons.

Nonetheless, the potential involvement of other reservoir hosts should also be considered. The wide range of bird species with evidence of USUV infection or exposure underscores the need to investigate their roles within the reservoir system, which may be critical for understanding USUV dynamics. For example, House Sparrows, in which we found evidence of infections in both live and dead individuals, were shown to be susceptible to USUV and able to transmit the virus to *Culex quinquefasciatus* in an American experimental study (Kuchinsky et al., 2022). House Sparrows species may therefore also contribute to the reservoir system of USUV and WNV in Western Europe. Corvids, including European species, have been shown to reach WNV viremia levels allowing transmission to mosquitoes, and are considered a highly competent reservoir host for WNV (Komar et al., 2003; Lim et al., 2015; Jiménez de Oya et al., 2018); some species may play a similar role in USUV ecology. In several species, we detected serological evidence of exposure to USUV without – or with only limited – concurrent molecular detections. This likely reflects the short duration of viremia relative to the persistence of antibodies, combined with lower sampling intensity of these species. Notably, herons, corvids, and raptors, for which evidence of exposure was found, have wider ranges of movement that cover extensive areas, and longer lifespans than Eurasian Blackbirds, and may play distinct roles in the transmission and spread of USUV within the reservoir system (Wit et al., 2024)."

4. L6: The authors say 'in recent years', could you be more specific please as this is quite subjective, and your description appears to capture c. 30 years

We have revised the sentence to clarify the time frame, it now reads: "Since the early 2000s, mosquito-borne arboviruses have increasingly expanded to and within Europe".

5. L6-8: Termed natural expansion? I believe it would be important to capture this to contextualise that these viruses do naturally expand and expansion is not solely driven by human activity.

We have revised a point in the description of contributing factors, it now read (now at L7): "i) the natural geographical expansion and establishment of viruses in resident bird and native mosquito populations".

6. L8: Regions in Europe with established *Aedes* species? If so please specify, as this could indicate areas outside Europe with invasive mosquito species.

We have revised this point to read: "outbreaks following the introduction of viruses by travellers into European regions with established invasive *Aedes* mosquito species".

7. L16: Use of 'their' for viruses to my mind is incorrect as it implies belonging, please edit to read as 'Culex pipiens mosquitoes are the main vector'

We have revised this sentence to read (now at L15): "In Europe, *Culex pipiens* mosquitoes are the main vectors."

8. L18: The authors mention these species have been particularly affected, to my mind this doesn't capture the true impact. For blackbird especially, USUV has been linked to disease mediated population declines. There are a number of appropriate references that show specific population declines of blackbird in areas where USUV has emerged or is circulating across Europe.

We have revised this sentence to read:

"Eurasian Blackbirds (*Turdus merula*) have been particularly impacted by USUV outbreaks in Europe, experiencing large-scale die-offs and population declines in affected areas" and we refer to studies showing population declines of Eurasian Blackbirds in Germany (Lühken et al., 2017) and the Greater London (Folly et al., 2020).

9. L25-26: 1% neurological symptoms – 1% of total infections (including asymptomatic), or 1% of the 20% that develop febrile illness? Please be specific.

We have revised this sentence to read (now at L26-28): "Most human infections with USUV and WNV are asymptomatic, however WNV can cause severe disease, with ~20% of infected individuals developing febrile illness, and up to 1% of infected individuals developing neurological symptoms."

10. L29: This statement implies that USUV spread from Austria, was Austria the first incursion (detected or not) of USUV? It is likely that other outbreaks had occurred previously, and these also contributed to geographic expansion across Europe. Could the authors rephrase this statement accordingly please.

We did not intend to imply a spread from Austria and have revised this section as follows (now at L30): "USUV was first detected in Europe in 2001, when it was identified as the causative agent of mass bird mortality in Austria (Weissenböck et al., 2002). Earlier presence was traced back to 1996 in Italy through retrospective analyses of samples from dead Eurasian Blackbirds (Weissenböck et al., 2013). Since then, USUV has been detected in most European countries (European Centre for Disease Prevention and Control European Food Safety Authority, 2023). Multiple lineages are present across the

continent, and phylodynamic studies indicate several independent introduction events from Africa into Europe, followed by subsequent evolution and spread within Europe (Engel et al., 2016).

While additional information is available regarding the probable directional spread of these lineages within Europe, we choose not to go into too much detail here as with the high genomic diversity, number of affected countries, as well as the uncertainty of the phylogeographic inferences and how these are likely biased by sampling effort, it would require a larger passage to describe what we know of USUV spread in Europe comprehensively.

11. L32: The authors claim that 'large' outbreaks occurred in humans and animals. This is quite a subjective statement, could the authors provide some context please, i.e. number of confirmed infections etc.

We have revised this passage to read (now at L36-40): "In 2004, WNV lineage 2 was detected in Hungary and rapidly became dominant on the continent. This lineage caused large outbreaks in humans and animals (Koch et al., 2024), including an outbreak in Greece in 2010 with 191 neuroinvasive human cases (Papa et al., 2011), and in 2018, the largest outbreak recorded in the EU/EEA, with 12 countries reporting 1,549 locally acquired mosquito-borne infections (Koch et al., 2024; European Centre for Disease Prevention and Control (ECDC), 2025).

12. L40: I disagree that circulation of one virus could influence the other. More appropriate to say that presence of one, may be an indicator that conditions are suitable for other arboviruses that have a similar ecological niche.

We agree that the presence of one virus may be an indicator that conditions are suitable for other arboviruses that have a similar ecological niche, we suggest this in the first part of our argument: "USUV circulation may indicate environmental suitability for WNV" (we here focus on the viruses of interest in our study and adapted this to the situation in the Netherlands, where USUV is present for a longer time, more widespread and more abundant than West Nile virus.)

However, we would like to maintain that beyond serving as an indicator of suitable conditions, the circulation of one virus could, in fact, influence the transmission dynamics of the other. Reviewer #1 expressed interest in this topic, and in response to their Major Comment 1, we have expanded on these interactions in the revised manuscript (Lines 307-317):

"However, given their overlapping transmission cycles, host and vector species, and antigenic relatedness, widespread USUV circulation may not only suggest the potential for increased local circulation of WNV – interactions between the two viruses in regions where they co-circulate may also occur. This possibility is supported by experimental studies: prior WNV infection in wild House Sparrows from the United States conferred protection against secondary USUV infection (Bosco-Lauth et al., 2024), while prior USUV infection in Domestic Geese reduced viral load and disease severity upon secondary infection with WNV (Reemtsma et al., 2023). In *Cx. pipiens*, prior exposure to USUV via infectious blood meals significantly reduced subsequent WNV infection and transmission, although WNV outcompeted USUV when mosquitoes were simultaneously exposed to both viruses (Wang et al., 2020). In the Netherlands, the broad host range, wide geographical distribution, and established enzootic presence of USUV may have impeded WNV transmission and spread following its emergence."

13. L46: 'Well before human or veterinary disease', please remove 'well', this is a subjective term that could be misinterpreted. I do agree however that detailed wildlife surveillance can detect emergence/outbreaks of arboviruses prior to human circulation.

Now at L54: We removed "well".

14. Figure 2: Is fantastic, well done, what an intuitive way to present the data.

We sincerely thank the reviewer for their kind comment.

15. L95-97: The authors say 'initially', I assume they mean in 2016, but could they please be specific when describing temporal data, as it is key for following the outbreak and understanding epidemiology. In addition, they give some region details, but perhaps some wider information on specific habitats could be useful – urban, rural, near wetlands etc and how that might link to their findings.

We have revised this sentence to read (now at L118): "Initially, in 2016 and 2017, USUV-positive birds were predominantly detected in the southern and central regions of the Netherlands, shifting to central and northern regions in 2018 (**Figure S1**)."

Regarding the additional habitat information, we agree that habitat context is important for understanding arbovirus dynamics. However a detailed analysis of habitat influences is beyond the scope of the current study. Our targeted and citizen science-based sampling was conducted across a wide range of habitats, and the sampling efforts were heterogeneous across studies (and for the live birds, across bird ringing locations), which makes this type of analyses more complex. Compiling and integrating data from these diverse sources was already a substantial undertaking, and a formal analysis of habitat influences would require a dedicated framework. That said, we fully agree that investigating habitat-related risk factors, while carefully accounting for sampling heterogeneity, is an interesting direction for future research.

16. L103-104: I would specify that this is RNA detection (I assume it is) and not just seroconversion as RNA implies either transmission is still occurring, or that viraemia in these hosts lasts longer than expected. Might be worth mentioning in main ms which host species and sample types these were.

We have revised this passage to read (now at L129-133): “However, USUV detections also occurred during the colder months: 14 live birds (12 Eurasian Blackbirds, 1 European Greenfinch -*Chloris chloris*-, and 1 Eurasian Coot -*Fulica atra*-) and 4 dead free-ranging birds (1 Eurasian Blackbird, 1 Fieldfare -*Turdus pilaris*-, 1 Eurasian Magpie -*Pica pica*-, and 1 Goldcrest -*Regulus regulus*-) tested positive for USUV RNA between December and February, inclusive.”

17. 105-106: Why were these species most frequently tested, a sampling bias due to mortality, for e.g., or targeted surveillance for key host species?

We have added information about this in the methods:

L379-386, under “**Citizen-science based sampling of live free-ranging birds**”

“Certain species were sampled particularly intensively, most notably Eurasian Blackbirds. This species is one of the most common breeding birds in the Netherlands (Sovon, 2020), is ubiquitous across habitats, and often occurs in human-associated environments (Lühken et al., 2017), making it relatively easy to capture in this study. Among the species abundantly sampled through the live bird study, Eurasian Blackbirds and Song Thrushes were proportionally more represented in serological testing. This may be attributed to their larger body size: since only up to 1% of a bird’s body mass can be safely drawn as blood, larger birds are more likely to yield sufficient serum for serological analysis. Additionally, bird ringers may be more hesitant to collect blood from smaller species.”

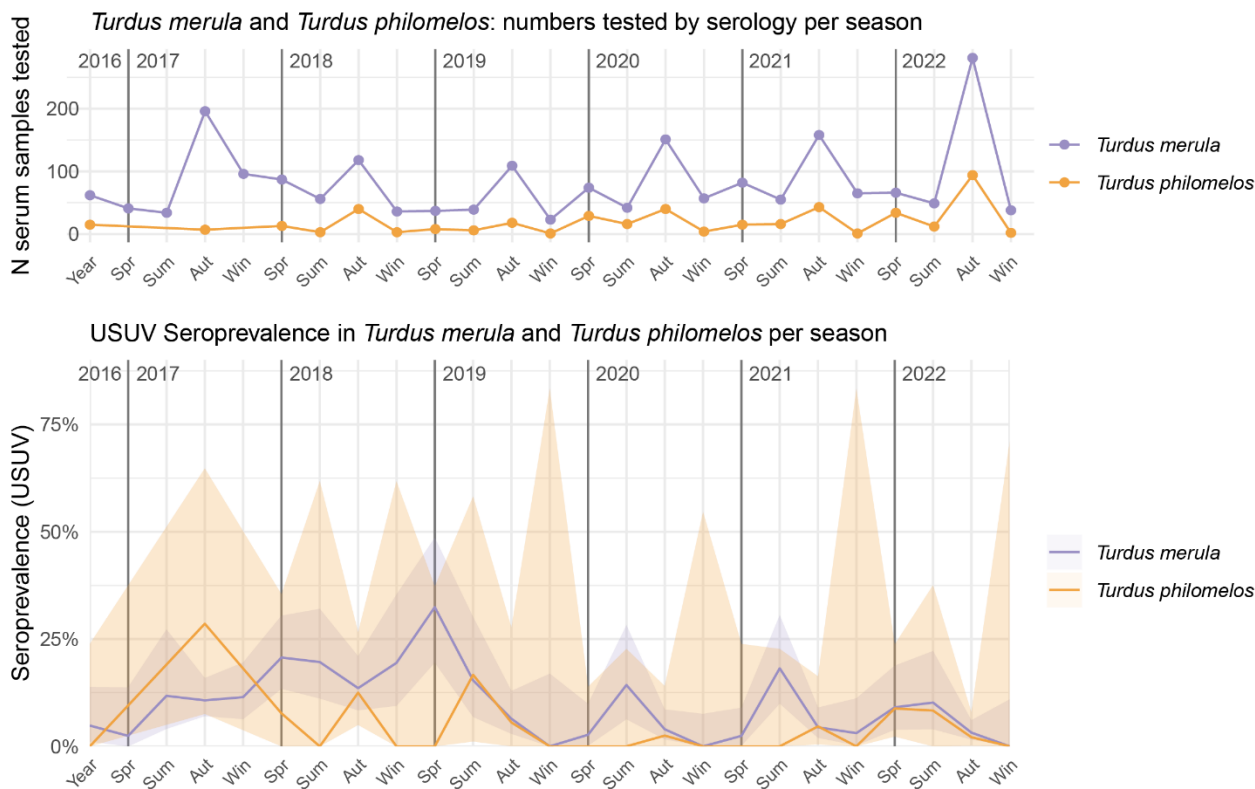
And L400-403, under “**Citizen science and zoological institution-based referral of dead birds for pathology**”

“Like in the live bird study, Eurasian Blackbirds were frequently represented in the dead free-ranging birds dataset. The characteristics described above (high abundance, broad habitat range, and proximity to human environments), combined with the high mortality associated with USUV in Blackbirds, likely contributed to their frequent submission through the citizen-based dead bird study.”

18. L108: ‘peaking at 26.7% in Eurasian blackbirds and song thrushes’ Could the authors confirm is 26.7% is the combined peak or both species had a similar individual peak? If the former (which I assume it is), could the authors further break that peak down into blackbird and song thrush specific values in the main ms as this would be helpful for understanding exposure in thrushes (one species highly susceptible, another not).

We had initially merged the serological data from Eurasian Blackbirds and Song Thrushes to increase sample size and temporal coverage. However, upon reviewing the data separately, we see that the broader temporal trends – including the 26.7% peak – are driven mainly by the Eurasian Blackbird data. This is something we had already observed when initially including all species, and our decision to focus on these two species was intended to account for this bias, but we can now acknowledge that this approach did not fully resolve the issue. Unfortunately, only 8 Song Thrushes were sampled during the season when this peak occurred, and none tested positive to USUV antibodies. Due to limited sample availability, separate species-level analyses do not allow for meaningful temporal trend extraction in Song Thrushes (see figure below for the reviewer's reference).

In response to this, we have revised the text to refer only to Eurasian Blackbirds in the relevant text (now L135) “An increase in USUV seroprevalence was observed from summer 2017, peaking at 32.4% in Eurasian Blackbirds in spring 2019, then dropping by autumn 2019 (**Figure 3B**).” and in Figure 3B.



19. L109: 'In 2016, serum testing was very limited' Could the authors explain why and quantify this statement.

We have revised this sentence to read (now at L140): "In 2016, serum testing was limited compared to subsequent years (3 positives/93 samples tested, or 3.2%), preventing confident conclusions about the geographical distribution of USUV-neutralizing antibodies." In addition, all numbers of samples tested and positive samples per research project and year are presented in Table S2.

20. L109-111: The seroprevalence data seems to mirror your molecular detections, might be worth explicitly saying that in the ms.

We have added explicit statements to this effect, they reads:

L137: "The temporal USUV seroprevalence patterns in Eurasian Blackbirds mirrored the USUV molecular prevalence trends, with higher seroprevalence observed in spring 2018 and spring 2019, following peaks in molecular prevalence in 2017 and 2018."

L144: "Notably, USUV-neutralizing antibodies were detected in regions where molecular detections had occurred during the preceding transmission season, supporting the pattern of viral spread from southern and central regions toward the north between 2016 and 2018, inclusive, followed by subsequent seroconversion in avian hosts."

21. L111-112: Why did serum sampling remain scarce?

We are limited by existing bird ringing station and individual bird ringers efforts at these stations, who all ring and sample birds on a voluntary basis. Unfortunately, the South is an area where sampling of live bird is limited (Figure S5) and as generally serum is collected only from a subset of captured birds, serum sampling is even more scarce.

22. L113: You use PMA abbreviation here and I don't believe I have seen it in the ms in full, only later in the methodology.

Thank you for noticing, we now introduced the abbreviation at first mention (L149).

23. Figure 4 and 5: Really lovely, informative panels, great!

We sincerely thank the reviewer for their kind comment.

24. L155: Over the last how many decades?

We have revised the sentence to clarify the time frame, it now reads (now at L193): "Since the early 2000s, the geographic range of arboviruses in Europe has expanded and these viruses have caused an increasing number of outbreaks in humans and in animals."

25. L158: You have talked about animal health previously, so this should read as, 'public and animal health and environmental strategies'.

We have modified the sentence to read (now at L194): "This emphasizes the need for a better, systemic understanding of their ecology and for the development of effective arbovirus monitoring approaches to inform public health, animal health and environmental strategies."

26. L167-168: This statement implies USUV infection in any bird often led to death, which is inaccurate. Primarily blackbird, with notable mortality recorded in some other groups, great grey owls, for e.g., could the authors amend this statement accordingly please.

We have modified the sentence to read (now at L209): "Because USUV caused substantial mortality among birds -particularly Eurasian Blackbirds-, monitoring the virus in citizen-reported dead birds proved especially valuable".

27. L246-247: Is WNV considered established in the Netherlands? I would tone this statement down, good evidence for USUV, less so for WNV. Also, the word phenomenon seems misplaced here. These arboviruses are emerging and persisting in temperate areas across Europe, so not really a phenomenon.

We have modified this passage to read (now at L368): "In conclusion, we document the emergence of two mosquito-borne arboviruses, USUV and WNV, in the Netherlands, a previously non-endemic country. Our findings show that USUV has been locally maintained for at least seven years with fluctuating transmission intensity, while WNV appears to be in an early stage of establishment."

28. Discussion in general: As per my major comment, the discussion is quite descriptive, without any major steps forward in our understanding. The authors have a unique and valuable dataset involving seven years of surveillance activity and I believe they could make more of their assumptions on arboviral epidemiology, or risk modelling.

We have addressed this point under **Major Comment 1** of Reviewer #3.