

Multiple species of native mosquitoes may be involved in Usutu virus circulation and maintenance in temperate zones

Robert Calam Bruce

calam.bruce@apha.gov.uk

Animal and Plant Health Agency

Anthony J. Abbott

UK Health Security Agency

Bathsheba L. Gardner

UK Health Security Agency

Amy C. Edwards

UK Health Security Agency

Harrison Hardy

UK Health Security Agency

Mirjam Schilling

Animal and Plant Health Agency

Andra-Maria Ionescu

Animal and Plant Health Agency

Ava J. Jenkins

Zoological Society of London

Mariana Santos

Zoological Society of London

Katharina Seilern-Macpherson

Zoological Society of London

Hugh J. Hanmer

British Trust for Ornithology

Robert A. Robinson

British Trust for Ornithology

Andrew A. Cunningham

Zoological Society of London

Becki Lawson

Zoological Society of London

Alexander G.C. Vaux

UK Health Security Agency

Arran J. Folly

Animal and Plant Health Agency

Jolyon M. Medlock

UK Health Security Agency

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Abstract

The introduction of emerging arboviruses into novel regions poses significant challenges for public, animal and ecological health. Usutu virus (USUV), first detected in the United Kingdom in 2020, has since become endemic in Southeast England. This study aimed to elucidate the role of native, temperate, mosquitoes in the transmission dynamics and maintenance of USUV. From March 2023 to February 2025 inclusive, 32,054 mosquitoes representing 21 species were collected from sites across central and southern England and screened for USUV RNA. Usutu virus RNA was detected in 116 (3.2%) of 3,637 mosquito pools across 13 species of five genera. *Culex pipiens s.l.* was the most frequently USUV-positive species, while the potential bridge vectors *Cx. modestus* and *Anopheles claviger* also exhibited notable prevalence. Phylogenetic analysis confirmed co-circulation of two lineages, Africa 3.2 and Europe 3, in multiple mosquito species within the same geographic regions. Finally, we show that prolonged periods of warmer weather could have promoted viral transmission and disease occurrence. These findings highlight the complexity of USUV transmission ecology in temperate zones, demonstrating the value of high-throughput vector screening for arboviruses to improve understanding of the underlying maintenance cycles of USUV circulation and better inform future risk assessments.

Introduction

Since the turn of the 21st century, the emergence and persistence of arboviruses, especially orthoflaviviruses such as Usutu virus (USUV) [1] and West Nile virus (WNV) [2], across the temperate zones of Europe have become a growing concern for public, animal and ecological health [3–5]. These processes are influenced by a range of biological, environmental and eco-social factors, including bird migration, increasing global trade and urbanisation, climate and land use changes and the expansion of invasive arthropod vector species [6, 7].

Effective surveillance of vectors is essential to underpin health risk assessments and guide interventions, while simultaneously improving our understanding of arboviral ecology in a changing world. The emergence and establishment of invasive mosquitoes, such as *Aedes albopictus* in mainland Europe [8–10], has been fundamental in facilitating recurring clusters of locally acquired arboviral infections, including dengue and chikungunya viruses, in people across multiple countries in the Mediterranean region, as well as in large cities such as Paris [11–13]. Furthermore, continued circulation of WNV in mainland Europe, likely driven by native mosquitoes, continues to result in substantial human, equine and avian cases each year [14].

In 2020, following expansion across mainland Europe, USUV was detected for the first time in the United Kingdom (UK) [15]. Initial passive surveillance efforts relied on opportunistic screening of dead wild birds to assess viral presence [15], supported by viral screening of mosquitoes at the index site [16]. Usutu virus was detected again in 2021 through both host and vector screening, and evidence of geographic expansion outside of Greater London was identified in 2023 [17]. Genomic analysis indicated that the 2021 detection was closely related to the 2020 emergence and unlikely to be a new introduction, suggesting that USUV could overwinter and persist in the UK [18]. Moreover, vertical transmission within *Culex pipiens s.l.* at the index site may also be contributing to local virus establishment [19].

Maintenance of USUV is facilitated through enzootic cycles occurring between mosquito vectors and avian hosts [20]. *Culex pipiens* biotype *pipiens* is often considered to be the primary enzootic vector of USUV among birds in Europe [21], and as such, vector surveillance across mainland Europe has predominantly focused on this species [16, 18]. However, while *Cx. pipiens pipiens* is primarily an ornithophilic species, other related taxa (such as *Culex modestus* and *Culex pipiens* biotype *modestus*) will feed on both avian and mammalian species, potentially acting as bridge vectors for human transmission [20].

While transmission of both USUV and WNV is associated primarily with the *Culex* genus, many other native mosquito species have been identified as either competent or potential vectors of flaviviruses across mainland Europe [22–24]. Elucidating native vector networks is essential to improve our understanding of emerging mosquito-borne virus epidemiology. Moreover, this information, coupled with a greater understanding of vector distribution, would help highlight high-risk regions susceptible to viral incursion and onward transmission, should an outbreak occur.

Recent studies have highlighted the potential of additional species, specifically *Cx. modestus* and *Culiseta annulata*, to act as vectors of USUV within laboratory conditions, although there is a paucity of field evidence [22, 25, 26]. Usutu viral RNA has been detected in a range of mosquito species across mainland Europe, including *Aedes vexans*, *Aedes caspius*, *Aedes detritus*, *Anopheles maculipennis s.l.* and *Anopheles plumbeus* [27, 28], although the extent to which these species are involved in USUV transmission is unclear. Given that all of these species are native to, and widely distributed throughout, the UK [29–31], and that USUV is now endemic in southeast England [32], there is a need to investigate the role they may play in the epidemiology of USUV.

Although symptomatic human cases of USUV infection remain relatively uncommon, infections have been associated with neurological disease and, in rare instances, fatalities in Europe [20, 33]. Additionally, USUV has caused substantial mortality in wild bird populations following its emergence across Europe, particularly in Eurasian blackbirds (*Turdus merula*), resulting in significant ecological impacts [15, 16, 20]. Furthermore, the emergence and establishment of USUV has frequently occurred alongside other zoonotic flaviviruses, including WNV, indicating shared ecological conditions that favour the emergence and persistence of mosquito-borne viruses more broadly [5, 23, 34].

In 2023, the Vector-borne RADAR (Real-time Arbovirus Detection And Response) project (www.vb-radar.com) was established to undertake a One Health approach to better understand the emergence, transmission and persistence pathways of mosquito-borne viruses, primarily USUV and WNV in wild birds, in the UK. Arboviral incursion is likely in wetland, rural, peri-urban and urban areas, so these were targeted for enhanced vector surveillance [35].

The current study aimed to enhance our understanding of the role of native mosquito populations in the maintenance of USUV transmission cycles in England. A deeper comprehension of potential vectors and their impact on arboviral transmission dynamics can highlight geographic regions and seasonal periods where virus transmission is more likely and inform robust and timely risk assessments. Moreover, vector surveillance combined with viral screening offer a high throughput approach to identify viral lineage distributions across large geographic areas and guide targeted active surveillance of avian hosts.

Methodology

Mosquito collection

Mosquito collection was conducted from March 2023 to February 2025 inclusive, utilising a range of active trapping methods at 30 sites across England (Fig. 1). Mosquito traps were deployed from May–October 2023 and June–October 2024 inclusive, and run every other week for four consecutive nights.

All mosquitoes from Cambridgeshire 2024 were captured using Biogents BG-Sentinel® (Biogents AG, Regensburg, Germany) traps and baited with BG-Lure® (Biogents AG) and CO₂ (CO₂ VB cylinder, 6.35 kg, supplied by BOC Ltd., Woking, UK). Mosquitoes in the Kent marshes were captured using both Mosquito Magnet® (MM) Executive traps (Woodstream Corporation, St. Joseph, MO, USA), using a propane cylinder (13-kg propane supplied by Calor Ltd., Warwick, UK) baited with an octenol (1-Octen-3-ol) lure (Woodstream Corporation) and BG-Sentinel® traps. Unless described above, all other mosquitoes collected were captured using Mosquito Magnet® with an octenol (1-Octen-3-ol) lure. In 2024 for both Kent and Cambridgeshire locations, traps were run bi-weekly for six nights and transported back from the field on dry ice. All identification of mosquitoes caught in Cambridgeshire and Kent was conducted on dry ice to preserve virus integrity. Pooled samples were frozen at -80°C until virus screening.

All mosquitoes were morphologically identified to species level using a stereomicroscope following dichotomous keys [36, 37]. All samples were pooled by species, sex, location and collection date with ≤ 13 mosquitoes per pool. Species that could not be distinguished by morphological identification alone were grouped as species complexes, hereafter referred to as species. Species complexes included: *Aedes annulipes* and *Ae. cantans* grouped as *Ae. cantans/annulipes*; *Aedes cinereus* and *Ae. geminus* grouped as *Ae. cinereus/geminus*; and *Culex pipiens s.l* and *Culex torrentium* grouped as *Cx. pipiens s.l./torrentium*.

Overwintering collections were conducted from December 2023 to February 2024 inclusive and January and February 2025 at ZSL London Zoo, the index site for the 2020 USUV detection. Resting or diapausing female mosquitoes in hibernacula were collected using a mechanical aspirator. Mosquitoes were then reared in an insectary for a maximum of three weeks at 25 °C and 50% relative humidity with 12:12 light cycles, while being fed a 10% sucrose solution every 4 days. This was undertaken to end dormancy and increase metabolic rates, aiming to promote viral replication to facilitate molecular detection. Mosquitoes were checked daily and dead individuals were stored at -80 °C for downstream molecular analysis [19]. All maps in this study were produced using QGIS Maidenhead (v3.36.1) [38].

RNA extraction

Nucleic acids were extracted from mosquitoes utilising one of three methods: (1) RNeasy Mini-prep (Qiagen, UK), (2) TRIzol™ chloroform precipitate (Invitrogen, Life Technologies Limited, UK) or (3) KingFisher™ Sample Purification System (Thermo Fisher Scientific, UK). Extraction methods varied according to equipment availability and laboratory requirements.

For Ribonucleic acid (RNA) extraction using the RNeasy Mini-prep protocol, mosquito pools were homogenised in 350 µl of Buffer RLT (Qiagen, UK) using a Qiagen TissueLyser II (Qiagen, UK) and a 5 mm steel ball bearing (Qiagen, UK). Extraction was then conducted in accordance with the manufacturer's protocol and RNA was eluted in two washes of 25 µl RNase free water (total elution volume of 50 µl).

For total RNA extraction using the TRIzol™ chloroform precipitate protocol, mosquito pools were homogenised in 300 µl tissue medium using a Qiagen TissueLyser II (Qiagen, UK) and a 5 mm steel ball bearing (Qiagen, UK) and centrifuged at 10,000 rpm for 10 minutes prior to TRIzol™ chloroform precipitate extractions. Total RNA was extracted from 250 µl of the supernatant, and the precipitated RNA was resuspended in 20 µl of RNase free water.

Finally, to facilitate high-throughput screening a KingFisher™ Sample Purification System was used. Prior to nucleotide extraction mosquito pools were homogenised in 500 µl of lysis buffer (Thermo Fisher Scientific, UK) using a Qiagen TissueLyser II (Qiagen, UK) and a 5 mm steel ball

bearing (Qiagen, UK) for 3 minutes. Nucleic acid extraction was conducted following the manufacturer's protocol and performed on a KingFisher™ Flex Purification System (Thermo Fisher Scientific, UK). Final nucleotide elution was conducted using 50 µl of elution buffer (Thermo Fisher Scientific, UK).

Comparisons of cold chain and non-cold chain samples were made by assessing the concentration and purity of extracted RNA. To ensure an accurate comparison, pools of 10x *Coquillettidia richardii* were selected from sites that utilised either cold chain or non-cold chain transportation methods, and were extracted by the same method (RNeasy Mini-prep protocol). This species was selected due to its wide distribution across multiple trapping sites, allowing comparable samples from both cold chain and non-cold chain collections. Extracted RNA was quantified using a DS-11 Spectrophotometer/Fluorometer (DeNovix Inc., Wilmington, DE) and differences in RNA concentration and purity were assessed using unpaired t-tests.

Viral screening using RT-qPCR and Sanger sequencing

All RNA extractions were subjected to USUV-specific RT-qPCR screens [39]. Positive amplifications were confirmed using a secondary RT-qPCR targeting Non-structural Gene 1 (NS1) [40] and the same cycling conditions [39]. All samples were also screened for WNV using established RT-qPCR protocols [41].

Mosquito pools that produced a positive USUV-specific RT-qPCR result were submitted for Sanger sequencing prior to phylogenetic analysis. Sequencing was conducted utilising a 3730 DNA Analyzer (ThermoFisher) and resulting chromatograms were viewed in MEGA-11 (v11.0.13) [42].

Bioinformatic analysis

Sequencing products were aligned and edited in MEGA-11 (v11.0.13) [42] to create a 168 bp alignment of the USUV NS1 gene including sequences generated in this study and 43 publicly available reference sequences (Supplementary Information S5) from Africa 3.2, 3.3 and Europe 3 lineages. Bayesian phylogenetic analysis was conducted in BEAST (v1.10.4) using a GTR + I nucleotide substitution model and 10 million Markov chain Monte Carlo generations. A 10% burn-in was conducted using TreeAnnotator (v1.10.4) and the resultant trees visualised in FigTree (v1.4.4). Nucleotide diversity (π), and segregating site (S) were calculated using MEGA-11 (v11.0.13) [42].

Mosquito-borne Viral Suitability Estimator (MVSE) Index P

Index P was calculated using the MVSE R-package [43], which estimates the suitability for mosquito-borne virus transmission based on climatic data and vector, host and virus specifics [43]. Daily peak temperatures, average humidity and precipitation were acquired for the South of England region from the Meteorological Office [44]. Priors were set to represent the most likely vector, in this case *Cx. pipiens sl.*, and USUV transmission in the UK (Supplementary Information S1), while index P values were visualised and plotted in GraphPad Prism (v10.2.3).

Results

A total of 32,054 individual mosquitoes, comprising 21 species from five genera, were collected (Supplementary Table S2). Of these, 61.3% (19,645/32,054) were collected in 2023, 37.1% (11,891/32,054) in 2024, and the remaining 1.6% (518/32,054) in January and February 2025.

The most speciose genus was *Aedes* (13 species, [mostly comprising 4 species: *Ae. vexans* (27.6% of *Aedes* spp. captured), *Ae. detritus* (23.4%), *Ae. cinereus* (19.6%), and *Ae. cantans/annulipes* (16.9%); $n = 12,246$]). *Culex pipiens s.l./torrentium* was the most abundant species complex caught, accounting for 26.4% (8,464/32,054) of all samples, followed by *Anopheles claviger* (12.7%; 4,060/32,054) (Supplementary Table S2). Mosquito numbers peaked from June to August in both 2023 and 2024, although the abundance of each genus varied (Supplementary Table S2). In both years, *Culex* species peaked in August ($n = 5915$ and 2565 respectively); *Aedes* numbers were relatively consistent in June and July 2023 and July 2024 ($n = 2041$, 2627 and 2638). *Anopheles* species (three species: 94.2% *An. claviger*) peaked in July and August in 2023 ($n = 956$ and 1067), although numbers were considerably lower in July 2024 ($n = 603$). Overall, the number of *Anopheles* specimens captured in 2024 were 71.7% lower than in 2023 ($n = 3357$ and 950 respectively).

Comparisons of *Coquillettidia richardii* pools, extracted using RNeasy Mini-prep (Qiagen, UK) and collected at cold chain and non-cold chain sites illustrated a significant difference between the two sample preservation protocols. Nucleotide extraction concentration and biological purity (A_{260}/A_{230}) were found to be significantly higher in cold chain samples ($p < 0.0001$), while chemical purity (A_{260}/A_{280}) was comparable between the two methodologies ($p > 0.05$) (Supplementary Figure S1 and Table S4). Furthermore, 38/58 (65.5%) of all Sanger sequences were generated from cold chain samples, supporting the improved RNA quality observed in these samples.

Virus-specific RT-qPCR screening detected USUV RNA in 116/3,684 (3.2%) of total mosquito pools from 13 species across five genera across both years. Of these, 50% (58/116) were corroborated through recovery of USUV RNA sequences by Sanger sequencing, while the remaining

samples did not produce reliable sequences for further analysis. Of the 58 Sanger-confirmed detections, 45 sequences were of sufficient quality for inclusion in phylogenetic analysis.

Culex pipiens s.l./torrentium had the highest number of infected pools, with 25/902 (2.8%) testing positive for USUV RNA, followed by *An. claviger* (24/453; 5.3%). Additionally, more than 10 positive pools were detected from *Cq. richiardii* (11/285; 3.9%), *Cx. modestus* (11/311; 3.5%) and *Aedes cantans/annulipes* (11/252; 4.4%). Proportionally, *An. plumbeus* (5/38; 13.2%), *An. maculipennis s.l.* (4/34; 11.8%) and *Cs. morsitans* (7/65; 10.8%) had the highest detection rates (Fig. 2B, Supplementary Table S3).

Of the 116 positive pools, 92 (79.3% of all positives) were detected in 2023 across 13 species of mosquito, 22 positive pools in 2024 across eight species, and the remaining two positive pools were detected in January 2025, both being *Cx. pipiens s.l.* (Fig. 3, Supplementary Table S3). Usutu viral RNA detection rates peaked in August in both years before declining through September and October. In 2023, USUV detections were made from May to October, and from June to October in 2024 (Fig. 2). Additionally, two pools of *Ae. vexans* tested positive for WNV RNA as reported previously [45].

Geographically, USUV was detected at 17 of 30 trap sites across the East Midlands and southern England. Twelve positive trap sites were located across Southeast England in Kent, Essex and Greater London; detections were also made at four sites in Cambridgeshire and at one site in Dorset (Fig. 4). Of these, detections were made in consecutive years at seven sites (41%), including the 2020 index site. The most northerly detection was 119 km north of the 2020 index site and was identified as lineage Europe 3, while the most westerly was 192 km west of the 2020 index site and found to be lineage Africa 3.2 (Fig. 4).

Phylogenetic analysis identified two USUV lineages circulating in our sampling locations: Africa 3.2 and Europe 3. Sequences within the Africa 3.2 clade had 100% identity to the NS1 gene fragment of the original 2020 UK detection ($\pi = 0$; $S = 0$). Sequences within the Europe 3 clade, first identified in samples collected in June 2023 were also highly conserved, with only four polymorphic sites observed ($S = 4$) and low nucleotide diversity ($\pi = 0.002$). Attempts to isolate viable virus and obtain longer sequence data through additional Sanger and Next Generation Sequencing (NGS) have been unsuccessful to date.

Mosquito-borne viral suitability analysis indicated differences in arbovirus transmission suitability between 2023 and 2024. Both years exhibited periods of high suitability (Index $P > 1$); however, 2023 experienced prolonged periods of high suitability, whereas 2024 showed more unstable periods. Peak P values were substantially higher in 2023 ($P = 38$), compared to 2024 ($P = 8.4$) across the south of England.

Discussion

In this study we demonstrate the value of high-throughput mosquito screening for arboviruses to understand viral epidemiology and inform animal and public health. A total of 32,054 mosquitoes, comprising 21 species, were collected from 30 locations and screened for USUV. Viral RNA was detected from across our sampling range in 116 pools comprising 13 different mosquito species, including four potential bridge vector species. Two USUV lineages (Africa 3.2 and Europe 3) were identified. While Africa 3.2 has been circulating and expanding throughout Great Britain since the 2020 index case [15–18, 46], the detection of Europe 3 indicated a second, previously unrecognised, incursion.

The detection of USUV RNA in a broad range of ornithophilic and mammalophilic mosquito species highlights the importance of conducting vector surveillance in different habitats throughout the mosquito active season to capture multiple potential vector species. Furthermore, our results suggest that multiple species of mosquito may be involved in USUV transmission cycles. Consequently, undertaking broad vector surveillance which targets a variety of species provides a deeper understanding of orthoflaviral transmission and persistence cycles in temperate climates. Although the detection of USUV RNA within a mosquito species does not demonstrate vector competence, our results suggest that USUV circulation may be more widespread in England than currently recognised, potentially involving undetected infection in both avian and mammalian hosts.

Three predominantly urban species of mosquito, *Cx. pipiens s.l./torrentium*, *An. plumbeus*, and *Cs. annulata*, tested positive for USUV RNA. Detection rates in pools of *Cx. pipiens s.l./torrentium* were comparable to those recorded from mainland Europe, where USUV is also endemic, and higher than observed previously in France [47]. *Culex pipiens* biotype *pipiens* is a ubiquitous, temperate, urban mosquito species and is regarded as the primary enzootic vector of USUV in Europe, which our data supports [21]. In the UK, this biotype is considered to be almost exclusively ornithophilic, whereas *Cx. pipiens* biotype *molestus* and *molestus/pipiens* hybrids are more opportunistic, feeding on both birds and mammals, including humans [48, 49]. Recent evidence suggests that the *molestus* biotype is not restricted to purely urban habitats, having been identified in rural and peri-urban areas [50, 51]. The wider distribution of *molestus* and hybrid bioforms may increase the geographic extent of human transmission risk to arboviruses more generally.

Other ornithophilic and mammalophilic vectors have been largely neglected regarding their involvement in *Orthoflavivirus* epidemiology. The range of mosquito species found to be positive for USUV RNA in this study indicates that many species are exposed to USUV and may contribute to aspects of its circulation across the urban-rural gradient. For example, *Anopheles plumbeus* is a tree-hole specialist, and its arboreal habitat could explain the high USUV detection rate in this species as it may feed on roosting or nesting birds. In addition, it can act as a bridge vector as it

bites humans and other vertebrates [52]. Although it is recognised as a nuisance biter of people in some areas, and is a competent vector of other arboviruses and *Plasmodium malariae*, *An. plumbeus* has not been demonstrated to be a competent vector for USUV [47]. *Culiseta annulata* is found throughout the country at low densities, exploiting a range of urban and wetland habitats, and is one of the most frequently detected British mosquitoes [29, 31]. Our results indicate that the detection rate in *Cs. annulata* was low compared to other species screened, which is not unexpected as this species is thought to primarily feed on mammals. However, it has been shown to be competent to transmit USUV in laboratory experiments in the UK [22] and the detection of USUV in wild populations highlights its potential impact on public and veterinary health. This species has been implicated in nuisance biting of people in areas it is found.

Four of the species with relatively high detection rates of USUV (*An. claviger*, *An. maculipennis s.l.*, *Cq. richiardii*, *Cs. morsitans*) are commonly associated with wetlands in the UK. They are not routinely considered human nuisance biting species, but are known as bird-biters, with *Culiseta morsitans* being exclusively ornithophilic. Birds can occur in high densities in both the breeding and migration periods providing ample opportunity for transmission. A further four mosquito species that were positive for USUV RNA are known to be nuisance biters of people or potential bridge vector species. Both, the *Ae. cantans/annulipes* group and *Ae. caspius*, are considered nuisance species in wet woodland and areas subjected to late summer grassland flooding respectively [53]. *Aedes detritus* is locally abundant in UK saltmarshes and estuaries, where it can be associated with considerable nuisance biting in adjacent communities, due to population blooms. However, its distribution and impact are highly localised and associated with summer flooded grassland [54, 55]. Whilst its generalist feeding behaviour may suggest a potential role as a bridge vector, its limited range and vector competence suggest that its overall contribution to USUV transmission in the UK is likely to be low [22]. The absence of USUV detections in *Ae. vexans* samples is surprising given it recently tested positive for WNV RNA in the UK [45], indicating ornithophilic activity within the same populations tested in this study. However, this may reflect low levels of USUV circulation within local avian populations at the study period.

Culex modestus does bite humans and is considered a major bridge vector for WNV, and a competent vector of USUV across Europe [22, 25]. Although reports of nuisance biting by this species in the UK are infrequent, presumably due to its patchy, highly localised distribution, we detected a relatively high USUV detection rate from May to October across both sampling years, indicating its potential to act as a bridge vector. In the Czech Republic, USUV RNA was detected in 0.15% ($n = 1/662$) pools of *Cx. modestus* [34], while a global review estimates *Cx. modestus* detection rates at 0.11% [33]. Therefore, where it is present in large numbers in the UK [30], it is likely that *Cx. modestus* presents a viable bridge vector for USUV and WNV. Consequently, surveillance for this species and defining its geographic range should be a priority when conducting any risk assessments for zoonotic flaviviral transmission.

The detection of USUV RNA in two pools of overwintering female *Cx. pipiens s.l.* at the 2020 index site supports recent findings of vertical transmission in *Cx. pipiens s.l.* at the same location [19] and indicates that USUV is capable of persisting through the winter in the UK, as observed in mainland Europe [56]. This provides a plausible mechanism for the persistence of USUV in Southeast England between transmission seasons and may contribute to its continued detection of USUV in Southeast England. Similar mechanisms also facilitate the establishment of future mosquito-borne arbovirus incursions in temperate zones.

The difference in sampling strategies (cold chain vs non-cold chain), not only highlighted that samples maintained on a cold chain system yielded higher total nucleotide concentrations and purity; but that USUV detections made in cold chain samples consistently produced more reliable sequencing results. This indicates that future sampling efforts should be conducted under cold chain conditions where possible.

Phylogenetic analysis identified two distinct USUV lineages within mosquito populations sampled in England, comprising the Africa 3.2 and Europe 3 lineages. Since its emergence in the UK in 2020, Africa 3.2 has been routinely detected through both mosquito and wild bird surveillance programmes [16, 17, 46, 57]. In contrast, evidence of Europe 3 circulation within the UK has been comparatively limited [57]. The detection of Europe 3 lineage viruses in multiple mosquito species across geographically dispersed sites in this study suggests that this lineage may be more widely distributed in England than previously recognised. Detections at four sites in Cambridgeshire and one in Dorset further support previous evidence from wild bird surveillance indicating a wider geographic distribution of USUV within England [17]. These molecular findings contributed to the recent assessment that USUV is endemic within Southeast England [32]. However, interpretation of phylogenetic relationships is limited by the short amplicon sequence available in this study, and higher-resolution genomic data will be required to better characterise lineage diversity, transmission dynamics and the origin of newly detected strains.

Mosquito surveillance for arboviruses may offer a high-throughput mechanism to identify viruses across large areas, complementing active and passive host surveillance [57]. Repeat USUV detections in both 2023 and 2024 were made at seven sites (across Dorset, Cambridgeshire, Essex, Kent and Greater London). However, higher resolution sequence data from future samples will be required to better determine the geographic origin of these incursions and differentiate between persistent local circulation and repeated introductions, which was not possible with the amplicon size obtained in this study.

Mosquito-borne viral suitability analysis indicated that 2023 was considerably more favourable for USUV transmission than 2024, driven by prolonged periods of elevated temperature and humidity, which are predicted to become increasingly common, at least in some areas of the country [58]. In contrast, 2024 was characterised by more variable conditions, including extended periods of rainfall and lower temperatures,

which may have disrupted mosquito population dynamics and reduced the duration of optimal transmission windows [61,62]. These differences were reflected in both mosquito abundance and the number of USUV RNA positive pools identified during this study. Elevated temperatures across Europe during summer 2023 were similarly associated with increased arbovirus transmission, although continued increases in WNV cases in 2024 highlight the complexity of climatic and ecological drivers influencing transmission dynamics [29,63]. The suitability index P represents transmission potential rather than realised transmission and cannot directly infer infection intensity without supporting entomological or epidemiological data [43]. In addition, the MVSE framework is primarily climate-driven and does not explicitly incorporate other determinants of transmission, such as vector abundance, host availability, or local ecological conditions [59]. Estimates are also sensitive to parameter assumptions, which may introduce uncertainty when applied to specific vector populations [43].

The findings in this study highlight the complexity of USUV transmission ecology in temperate areas and the value of undertaking broad vector surveillance targeting multiple species across a range of habitats to inform epidemiology. Our results show that while *Cx. pipiens* s.l. is indeed an important enzootic vector, relatively high detection rates in multiple other ornithophilic and mammalophilic mosquitoes indicate that a broader range of species may contribute to viral maintenance and should be considered when assessing zoonotic transmission risk.

Declarations

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Data availability:

All nucleotide sequences generated in this study have been submitted to the National Center for Biotechnology Information (NCBI) GenBank database and are awaiting assignment of accession numbers. Accession numbers will be provided upon acceptance or once assigned.

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Author Contributions:

Project design: BL, RAR, AAC, JMM, AGCV and AJF. Sample collection and identification: AJA, BLG, ACE, HH, AJJ, MS, KSM, AGCV and JMM. Molecular laboratory methods: RCB, MS, AMI and AJF. Molecular Analysis and bioinformatics: RCB, MS, AMI, HJH and AJF. Resources: AGCV, JMM and AJF. First draft: RCB, AJF and JMM. Final review and editing: All authors. Funding and supervision: RAR, AAC, BL, JMM and AJF.

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Figures

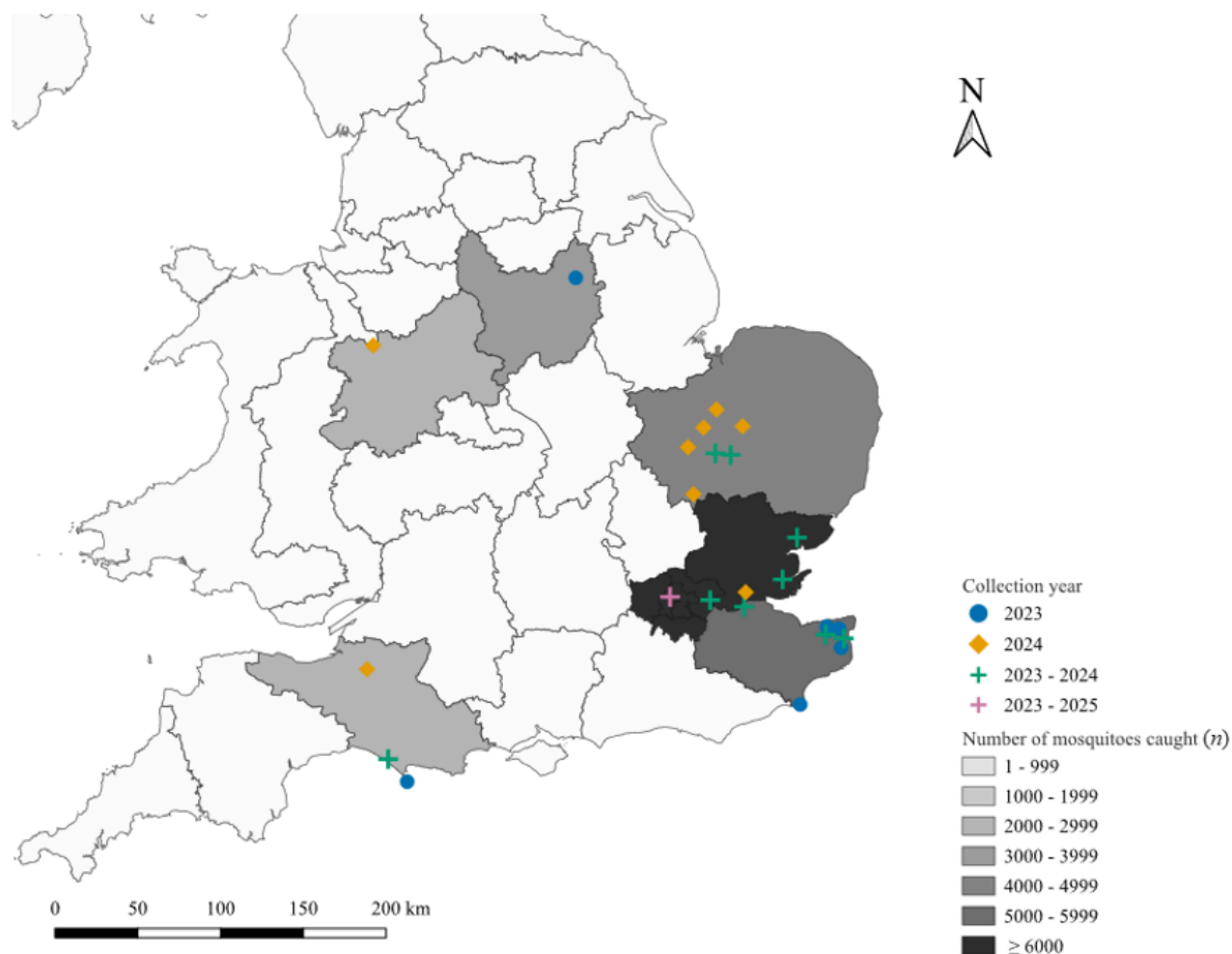


Figure 1: Geographical distribution of mosquito surveillance sites ($n = 30$) for flavivirus screening (Supplementary Table S1). Trap locations and year deployed are shown by colour and icon (● = 2023 sampling sites; ◆ = 2024 sampling sites; + = sites sampled in 2023 and 2024; + = site sampled in 2023, 2024 and 2025), the shaded regions illustrate the number of mosquitoes collected in each region, March 2023-February 2025 inclusive. Due to the close geographical proximity of some sampling locations in Kent are overlapping on the map but can be identified in Supplementary Table S1.

Figure 1

See image above for figure legend.

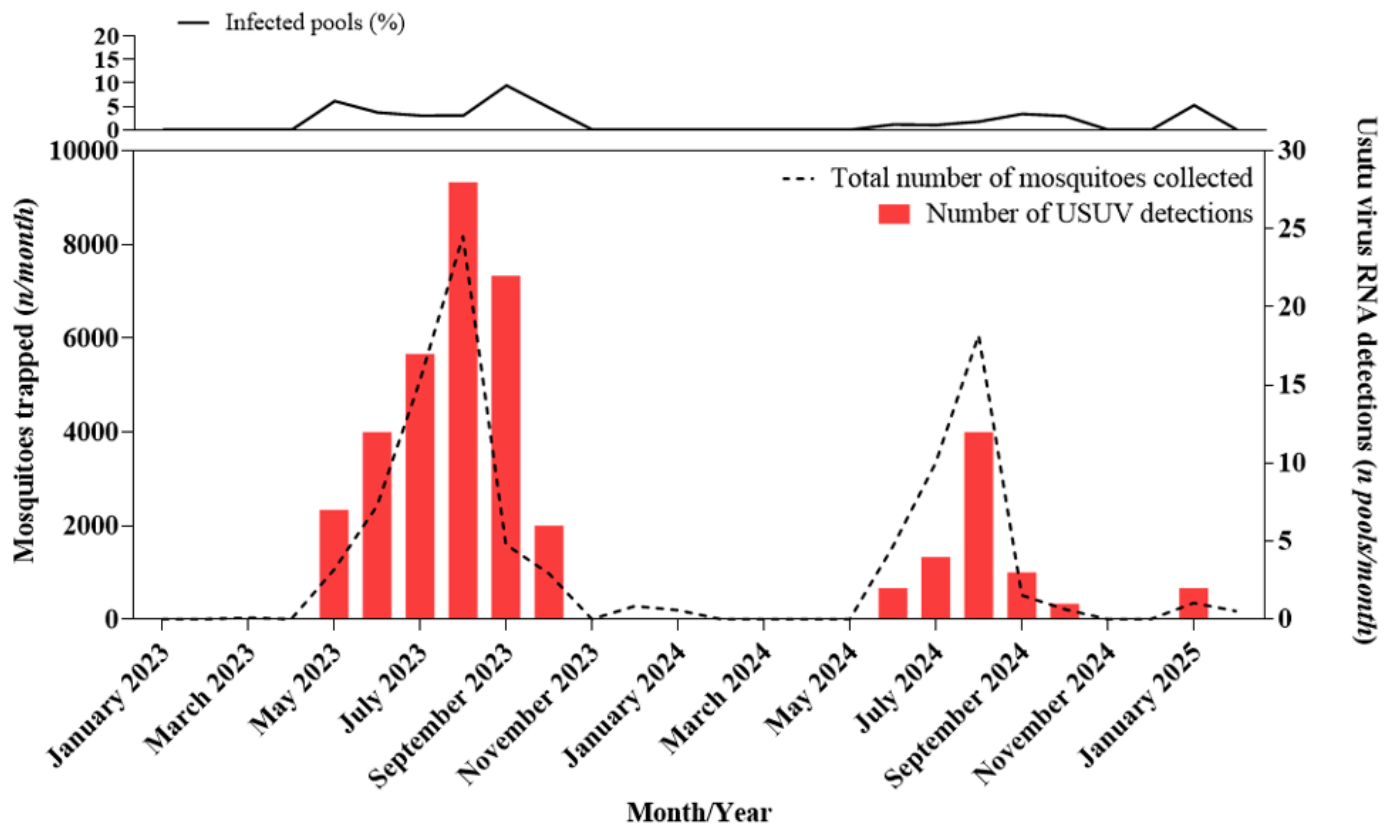


Figure 2

The detection rate of Usutu virus (USUV) positive mosquito pools/month (top), illustrating the average detection rate across all pooled samples. The overall number of mosquitoes collected each month (left Y axis) and the number of mosquito pools testing positive for USUV RNA (right Y axis) (January 2023 to February 2025 inclusive) (bottom). Visualised in GraphPad Prism (v10.2.3).

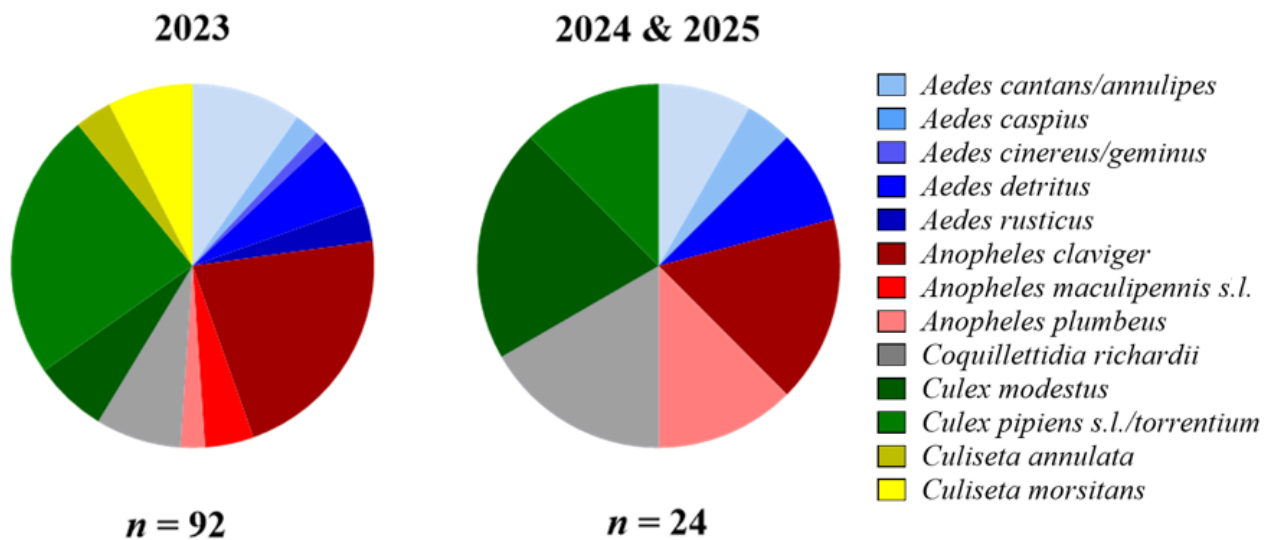


Figure 3

Composition of mosquito species in which Usutu virus (USUV) RNA was detected during the study (n is the number of positive pools). The detection of USUV RNA in two pools of overwintering *Culex pipiens s.l.* females in January 2025, have been included with the 2024 detections.

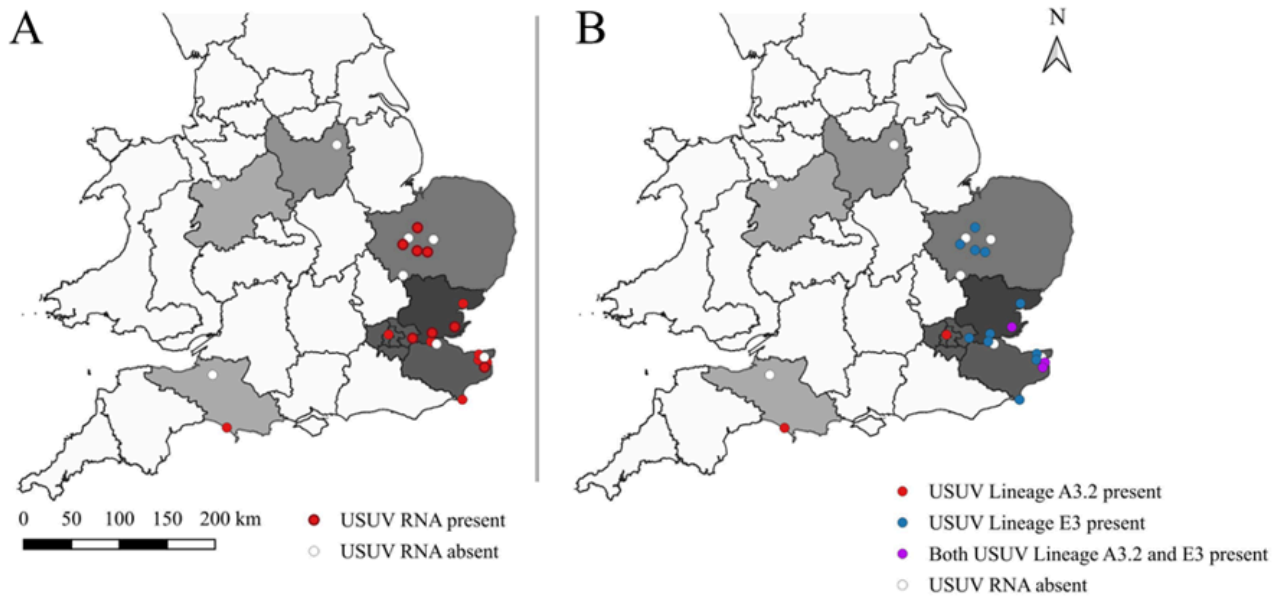


Figure 4

Geographical distribution of Usutu virus (USUV) RNA detection in mosquito populations at sampling sites across southern and central England, March 2023 to March 2025 inclusive. Shaded regions illustrate the number of individual mosquitoes screened (Fig. 1), regional boundaries are illustrated at NUTS2 level. A) Red icons illustrate sites where USUV RNA was detected; white icons indicate no detection. B) The lineage of USUV as determined by Sanger sequencing. Red points indicate sites where only Africa 3.2 (A3.2) was detected, while blue indicates sites where Europe 3 (E3) was detected. Purple sites indicate that both Africa 3.2 and Europe 3 lineages were detected.

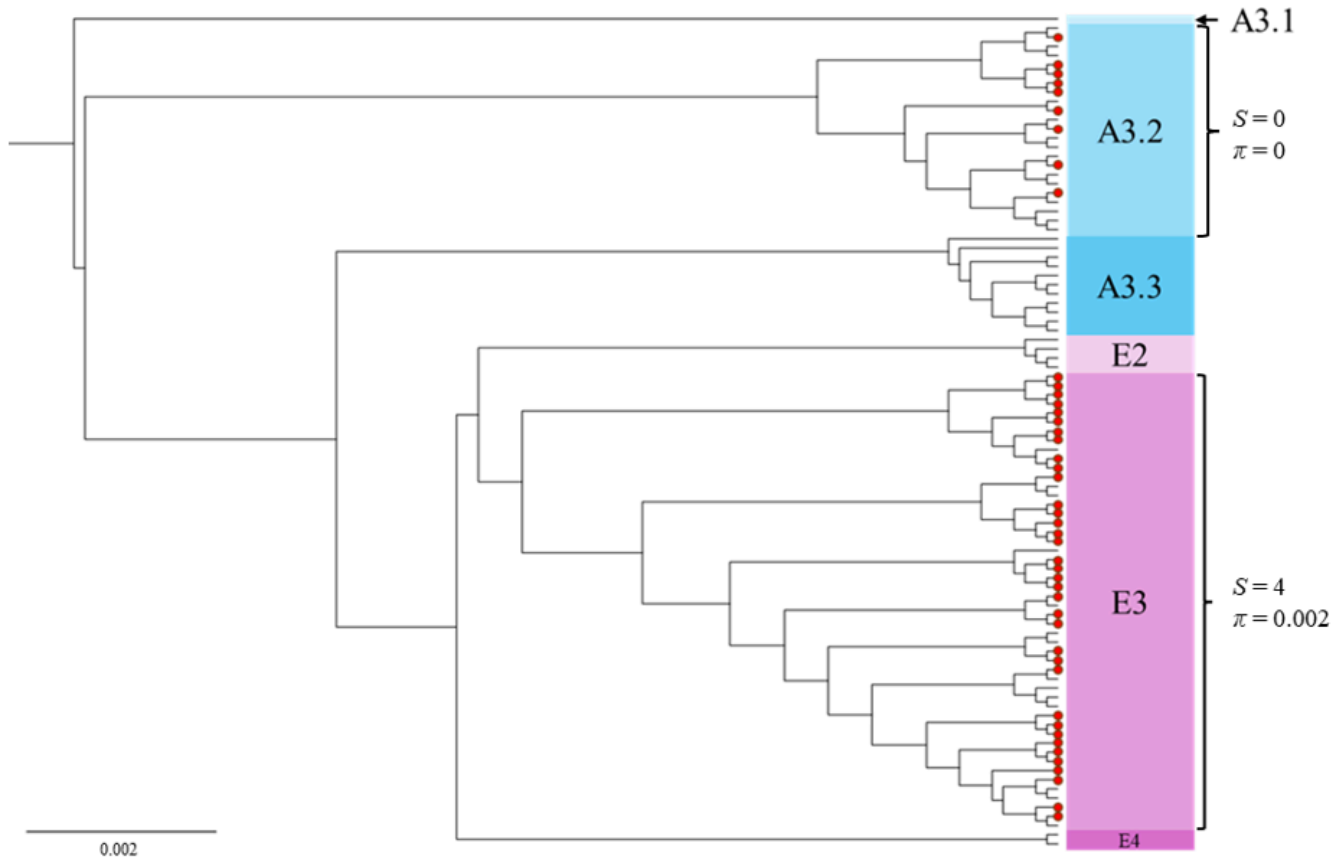


Figure 5

A Bayesian phylogenetic tree based on the 168 base pair fragment of the Usutu virus (USUV) non-structural protein 1 (NS1) gene obtained through Sanger sequencing, alongside 44 USUV reference sequences from the Africa 3.1, 3.2, 3.3 and Europe 2, 3 and 4 lineages (A3.1, A3.2, A3.3, E2, E3 and E4 respectively). The nucleotide diversity (π) and the number of polymorphic sites (S) are indicated. The red circles denote sequences generated in this study, branch length is shown proportional to the scale bar of 0.002 nucleotide variations per site.

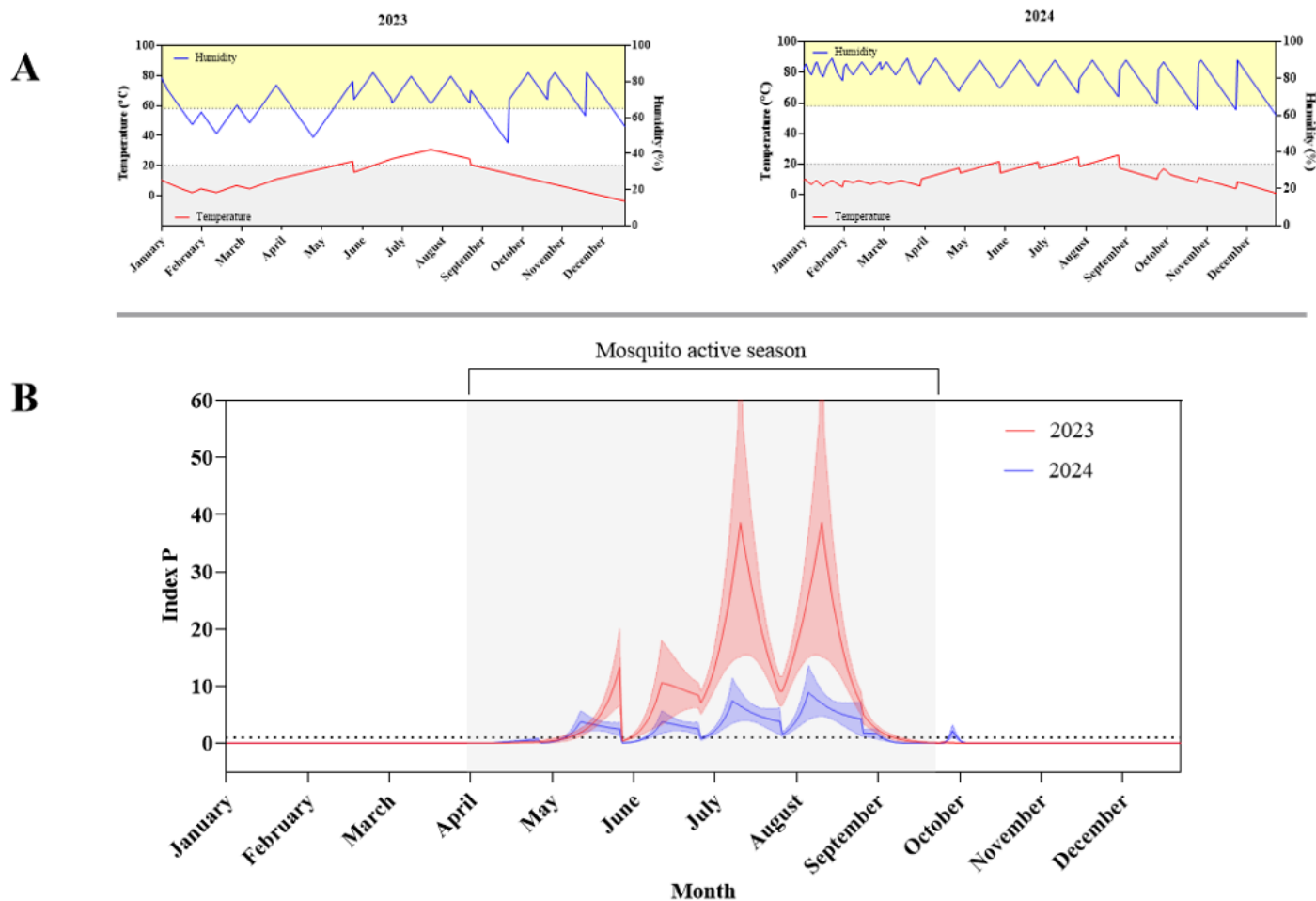


Figure 6

A) Peak daily temperature and humidity values across Southeast England 2023 and 2024. Grey shaded regions represent periods with temperatures < 20 °C, while yellow shaded regions indicate periods with humidity > 65%. Periods when temperature exceeds 20 °C and humidity is above 65% correspond to higher vector suitability.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1.xlsx](#)
- [MultiplespeciesofnativemosquitoesmaybeinvolvedinUsutuvircirculationandmaintenanceintemperatezonesSupplementaryInformation.docx](#)