

Honey-baited FTA cards in box gravid traps for the assessment of Usutu virus circulation in mosquito populations in Germany

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ABSTRACT

Usutu virus (USUV) is becoming increasingly important to veterinary and human health in Germany. USUV has been implicated in mass die-off events of birds, especially of blackbirds (*Turdus merula*), and has experienced significant range expansion in the years since its first detection in 2010. Current detection methods rely primarily on dead bird surveillance or mass mosquito collection using CO₂ as the main attractant. Dead bird surveillance can result in detection of disease circulation past the point at which control efforts would be most impactful. Vector surveillance offers the opportunity to detect disease circulation before significant outbreaks occur. However, current methods result in collections of extremely large numbers of predominantly nulliparous female mosquitoes who have not yet taken a blood meal. This study sought to test whether box gravid traps could successfully trap USUV infected gravid *Culex* mosquitoes, and if viral RNA could be successfully transferred and stabilised on an FTA card. During the month of August 2020, 18 Reiter-Cummings style box gravid traps with honey-baited FTA cards were set in a region of known USUV circulation around the southern border of Hesse, Germany. Four 48-hour trapping rounds were conducted. All mosquitoes and FTA cards were collected and stored during transport to the laboratory on dry ice. Samples and FTA cards were then transferred and stored in a freezer at -5 °C until identification. Identification was carried out on a chill plate before being sent with overnight courier in a styrofoam box with cooling elements for virus detection with a modified generic flavivirus RT-PCR. Mosquitoes were separated into pools by trap, date, species and feeding status. 2003 mosquitoes were caught in four rounds of trapping, 1834 or 88% of which were female *Culex* mosquitoes used for examination. 13 pools of mosquitoes and four FTA cards tested positive for USUV. No positive FTA cards were found in traps with positive mosquitoes and no positive mosquitoes were found in traps with positive FTA cards. Although fewer FTA cards than expected returned a positive result, this may have been a result of the extreme conditions experienced in the field and highlights the need to establish the temperature and humidity boundaries such a collection method can withstand. Box gravid traps however, provided a highly effective and targeted approach for capturing gravid female *Culex* mosquitoes, the most appropriate subpopulation for testing for USUV. Additionally, the simplicity and effectiveness of this trapping and surveillance method make it an attractive option for use as an early warning system, including for large scale surveillance programmes.

Abbreviations: EVS, encephalitis virus surveillance; FTA, Flinders Technology Associates; IgG, Immunoglobulin G; IgM, Immunoglobulin M; KABS, Kommunale Aktionsgemeinschaft zur Bekämpfung der Schnakenplage eingetragener Verein; RT-PCR, Reverse transcription polymerase chain reaction; USUV, Usutu virus; WNV, West Nile virus; RNA, Ribonucleic acid.

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1. Introduction

Usutu virus (USUV) is a member of the family *Flaviviridae* and belongs to genus *Flavivirus*, with eight lineages grouped by geographic origin (Roesch et al. 2019; Cadar, et al., 2017). It is closely related to other members of the Japanese encephalitis virus complex which includes Murray Valley encephalitis and West Nile virus (Roesch et al., 2019). It is typically transmitted by *Culex* mosquitoes, with the main vector in Germany being *Culex pipiens* s.l (Becker et al., 2012; Roesch et al., 2019; Gould and Solomon, 2008). Other species are considered to play a more minor role.

First recorded in South Africa in 1959 from *Cx. neavei* mosquitoes (Calisher and Gould, 2003; Gould and Solomon, 2008), it was originally thought to be unimportant for veterinary health but has subsequently been shown to be highly pathogenic to several bird species (Rubel et al., 2008). Humans are considered dead-end hosts for USUV showing low levels of viraemia (Clé et al., 2019). Whilst it was assumed that it had a low pathogenicity to mammals including humans (Weissenböck et al., 2003), there is a growing body of evidence which shows that the virus in individual cases can result in illness in humans (Calzolari et al., 2012). Clinical cases of USUV have been reported from Italy (Pecorari et al., 2009), Croatia (Santini et al., 2015), France (Simonin et al., 2018), and Hungary (Nagy et al., 2019), with two asymptomatic cases detected so far in Germany through blood donor screening (Allering et al., 2012; Cadar et al., 2017).

In an area with endemic USUV in Italy, a retrospective analysis of cerebrospinal fluid and serum samples taken from patients both with and without neurological symptoms found a seroprevalence rate of over 6% in just 609 individuals, showing that human infections are not rare and can result in disease of the central nervous system (Grottola et al., 2017).

Many arboviruses, including USUV, have experienced marked expansion in recent years, resulting in epidemics in regions which had previously never been affected, such as is the case for Europe (Gould and Higgs, 2009; Pfeiffer and Dobler, 2010). USUV has spread extensively since its first detection within Europe (Weissenböck et al., 2013; Clé et al., 2019) and continues to spread northwards through Germany with evidence for the recent introduction of new lineages (Michel et al., 2019). At least four strains have now been detected in Germany (Cadar et al., 2017).

USUV is maintained in an enzootic cycle between *Culex* mosquitoes and birds (Grottola et al., 2017) with passerines, especially blackbirds (*Turdus merula*), house sparrows (*Passer domesticus*), and Strigiformes including the Great Gray Owl (*Strix nebulosa*) functioning as amplifying hosts (Clé et al., 2019; Jöst et al., 2011; Becker et al., 2012).

The highest numbers of USUV positive birds in Germany have been found between June and September (Ziegler et al., 2015) and is directly associated with high summer temperature conditions (Cadar et al., 2017). Although blackbirds seem to be the species most vulnerable to USUV, it is likely that deaths and effects in other species are at least somewhat underestimated, and recognition of blackbird die-offs is partly due to their conspicuousness in urbanised areas (Ziegler et al., 2015).

Pathogen surveillance highly depends on the possibility to catch appropriate numbers of potentially infected mosquitoes as well as the requirement for specialist staff and rapid but reliable virus detection methods (Engler et al., 2013). Both Ritchie et al. (2007) and Lindsay et al. (2001) outlined the need for a more efficient system to overcome many of these issues by bypassing the collection of vast number of live mosquitoes.

After finding that *Culex* mosquitoes will expectorate enough saliva onto a sucrose infused cotton pad for virus detection, van den Hurk et al. (2007) hypothesised that such a method could find application in the field as part of a surveillance system, where identification of the vector is not necessarily required and where timely detection of virus is paramount. From the discovery, a mosquito-free surveillance system was

developed, the first versions of which used CO₂ updraft traps with honey-baited Flinders Technology Associates® (FTA) cards, later versions using CO₂ baited passive traps (van den Hurk et al., 2012; Ritchie et al., 2013), and most recently, infusion baited box gravid traps (Wipf et al., 2019).

Hall-Mendelin et al. (2010) were the first to implement the method with an updraft trap, which offered mosquitoes honey on a nucleic acid preserving substrate, such as an FTA card. Any RNA expectorated onto the cards is inactivated and preserved for several days and can be detected with real-time reverse transcription polymerase chain reaction (RT PCR).

This study sought to determine the suitability of using box gravid mosquito traps alongside honey-baited FTA cards as an alternative to current mosquito surveillance methods for USUV in Germany. Current methods are time consuming, expensive and target the subpopulation least likely to be infected with these viruses.

The aims of this study were as follows. The first was to determine the most effective attractant for use with the box gravid traps. Second, to test self-made gravid traps in their ability to collect gravid and USUV-infected *Culex* mosquitoes. Third, to evaluate if mosquitoes will feed on the FTA cards and lastly, to ascertain if the FTA cards preserve viral RNA under the conditions presented in the field in Germany.

2. Materials and methods

2.1. Trap construction

The gravid traps were based on the original design by Reiter (1983) and the Reiter/Cummings style gravid trap (Cummings, 1992). Components, construction and use followed the method outlined in Fynmore et al. (2021) with a Whatman™ FTA™ Classic cards (GE Healthcare Life Sciences, Buckinghamshire, UK) (FTA cards) cut into quarters and soaked face down overnight on 1:100 blue-dyed honey ((Brilliant Blue FCF (Blue 1) food colouring powder (Brillantblau FCF (C.I. 42090), Carl Roth GmbH & Co. KG, Karlsruhe, Germany); Goldland Blütenhonig, W. L. Ahrens GmbH & Co. KG, Paderborn, Germany;). This was then inserted into a 3 × 4 cm resealable sample bag with 1.5 cm square hole cut in the front and half of a cotton pad (dm-drogerie markt GmbH & Co. KG, Karlsruhe, Germany) inserted behind this. The cotton pad was soaked at the time of deployment in a 1:10 ratio of blue-dyed honey and water to keep the FTA card moist during trapping.

2.2. Attractant development and selection

Water-based infusion attractants were tested in combination with the gravid traps to determine the most effective attractant for collecting *Cx. pipiens* s.l./*Cx. torrentium* in the area. Four common combinations were tested, with proportions and method closely following Ritchie (1984) and Reiter (1983). The first was a basic hay infusion made with a ratio of 0.9 kg of hay (Aktiva Langhalm-Stroh; Aktiva Heimtierprodukte GmbH & Co. KG, Elmshorn, Germany) steeped in 114 l of water for seven days. The second was the same basic hay infusion with the addition of 10 g of brewer's yeast (Bierhefe; Dr. Hesse Tierpharma GmbH & Co. KG, Hohenlockstedt, Germany). The third was basic hay infusion with brewer's yeast with 99.9% isopropyl alcohol (Fürth Chemie GmbH, Leipzig, Germany) added in a 4:1 ratio as described by Ritchie (1984). The fourth was hay infusion with 10 g brewer's yeast and 5 g of pure whey protein (lactalbumin) (Sports Supplements Ltd. (Bulk Powders), Colchester, United Kingdom).

Hay infusion was brewed following a similar procedure to Ritchie (1984) with modifications for containment of the hay. For each infusion, hay was added to two fine holed aquatic plant baskets, wired shut together, and the closed basket fixed to the sides of a rainwater barrel with garden wire for ease of removal. Baskets were agitated in the water daily. Brewer's yeast and lactalbumin were added directly into the water where required. Each infusion variant was brewed in a covered area

outdoors in separate barrels to eliminate cross contamination with the barrels covered with insect screen to prevent mosquitoes from accessing the infusion. Isopropyl alcohol was only added upon setting the trap in the field. At the end of the seven-day period, the hay in the basket was removed and the infusion was ready for use.

A Latin square was set up with four traps containing the four different attractants labelled as treatments 1, 2, 3 and 4. These four treatments were rotated throughout four positions labelled A, B, C, and D, 35 m away from one another over four days. Four rounds were carried out creating a total of 16 datapoints for each trap. The position for each treatment type for each round was randomised, so that each treatment was randomly placed once at each of the four positions. In the case that a trap was not functioning on one of the days, that day's setup was repeated.

2.3. Site selection

The detection of USUV from mosquitoes trapped in 2010 were from mosquitoes caught in gravid traps in an urban environment, where the density of key vector (*Cx. pipiens* s.l./*Cx. torrentium*) is high and host species (Eurasian blackbird *Turdus merula*) are more common than in forested or agricultural areas (Jöst et al., 2011). Based on this, 18 sites were selected to cover a small region with known USUV circulation from previous years in urban to peri-urban areas.

Within this region, sites were selected based on reports of *Culex* activity or dead birds (mainly *Turdus merula*) sightings reported to KABS, with opportunistic placement in locations which were hidden from public view or on private property to reduce the risk of tampering or theft. At each location, traps were placed in semi-sheltered areas in partial shade where possible.

2.4. Field deployment and field data collection

Before initial deployment, traps were left outside for two weeks to minimise the risk of volatile compounds having a deterrent effect on the mosquitoes. Traps were numbered by location and used only at their designated locations.

A total of four trapping rounds were conducted in the month of August 2020. Fresh simple hay infusion (treatment 1) was used for each deployment date. The traps were set for 48 h (Figs. 1 and 2), and samples collected and processed following the procedure outlined in Fynmore et al. (2021) but with mosquitoes from individual traps pooled by species and feeding status in groups of up to 50 specimens.

Mosquitoes were identified to species using the keys from Becker et al. (2010) and Tanaka et al. (1979) under stereo microscope (Zeiss Stemi 508; Carl Zeiss Microscopy GmbH, Jena,

Germany) and inspected for the presence of blue colouring. *Culex torrentium* was not separated from *Culex pipiens* s.l because females are not able to be differentiated morphologically (Rudolf et al., 2013). Care

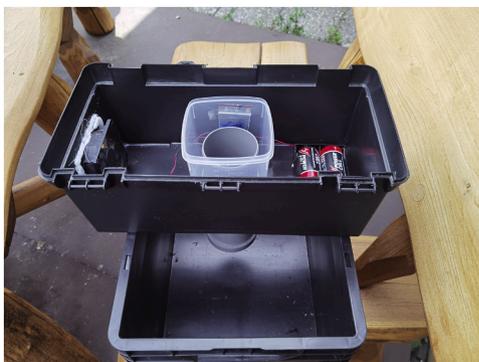


Fig. 1. Example picture showing open trap with FTA card inside collection container.



Fig. 2. Example pictures of traps deployed in the field showing hay infusion in the lower tray.

was taken to ensure any blue seen was internal to the mosquito rather than merely transferred externally on the mosquito body. Additionally, the numbers of mosquitoes already dead upon collection and number appearing gravid was recorded. By-catch was discarded, and males were discarded after identification.

Temperature data for use in the statistical analyses were downloaded for the closest weather station (Darmstadt) from the German Weather Service (Wetterdienst, 2021).

2.5. RNA extraction and PCR protocols

Mosquito specimens were pooled by trap, sampling date, species, and feeding status, determined by the observation of blue colouring in the digestive tract.) There were a maximum of 50 specimens per pool with samples exceeding this size split into two or more pools. Mosquito specimens were placed into 2 ml tubes, and about 10 pieces of 2.0 mm zirconia beads (BioSpec Products, Bartlesville, USA), as well as 0.5 ml of cell culture medium (high-glucose Dulbecco's modified Eagle's medium; Sigma-Aldrich, St. Louis, MO, USA), was added. The homogenisation was performed with a TissueLyser LT (Qiagen, Hilden, Germany) for 2 min at 50 oscillations/s.

FTA cards were individually placed into 2 ml tubes with 1 ml phosphate-buffered saline (PBS; PAN Biotech, Aidenbach, Germany). The tubes were kept on ice for 30 min and vortexed three times. We used 200 μ l of the mosquito homogenates and PBS solution of the FTA cards for RNA extraction, which was performed with KingFisher™ Flex Magnetic Particle Processor using MagMAX™ CORE Nucleic Acid Purification Kit (both Thermo Fisher Scientific, Waltham, MA, USA).

Samples were tested for flavivirus RNA using a modified generic flavivirus reverse transcription (RT)-PCR (Becker et al., 2012). All amplicons were visualised on 2% agarose gels and PCR products sequenced with LGC Genomics (Berlin, Germany). Sequences were visualised and edited with Geneious version 9.1.7 (Biomatters, Auckland, New Zealand). The resulting sequences were submitted for virus species identification using the Basic Alignment Search Tool (BLAST) in the GenBank DNA sequence database (<https://blast.ncbi.nlm.nih.gov/>).

3. Statistical analyses

All data were analysed using IBM SPSS Statistics 25 (IBM Corp., 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, n.d.) with the significance level set to $p < 0.05$. To compare which infusion bait was most suitable for use in the field, a one-way ANOVA was performed on the Latin square data to compare catch sizes obtained from each infusion. Total numbers of females caught per trap in the Latin square trapping runs were log transformed ($\log + 1$) to reduce skew and kurtosis and create a normally distributed data set for analysis.

Temperatures throughout the trapping periods were assessed for normality with a Kolmogorov-Smirnov test and the mean temperatures

for each period assessed using one-way ANOVA with a Tukey post-hoc analysis and Bonferroni adjustment. This highlighted a significantly higher mean temperature of one 48-hour sampling period to the remaining three sampling periods. Subsequently, temperature data was redefined as a categorical variable with a binary outcome; 48-h average temperatures above 26°, and 48-h average temperatures below 26°, with 26° being the upper threshold of three sampling periods. This binary data was used for all calculations involving 48-h average temperature.

The number of females mosquitoes caught in the field was also investigated for normality and log transformed. Likewise, the percentage of mosquitoes deceased upon collection also required transformation and an arcsine square root transformation was performed. Only the transformed data were used in statistical tests.

Independent samples t-tests were run to assess for influence of the categorised temperatures on the numbers of mosquitoes caught per trap as well as the percentage deceased upon collection, and to check for a relationship between the number of mosquitoes which had fed in the trap and the likelihood of a trap being positive or negative for virus.

A linear regression was performed between the percentage of fed mosquitoes and the number of mosquitoes caught per trap.

A Fisher's exact test was run to investigate a possible relationship between the 48-hour average temperature and a virus positive or negative trap, a relationship between 48-hour average temperature and an FTA card being positive or negative, and sun exposure of each location and FTA card result.

4. Results

4.1. Latin square for infusion selection

A total of 412 mosquitoes were caught, of which 404 were identified as females. 93.8% (n = 379) of these females belonged to the genus *Culex*. The remaining females belonged to genus *Aedes* (n = 22) and genus *Anopheles* (n = 3). Male mosquitoes amounted only to 1.9% of the sample (n = 8). The average number of mosquitoes caught per trap night across all traps was 6.4. The maximum number caught was 24. Of the female *Culex* mosquitoes, 84.7% (n = 321) appeared to be either gravid or had fed at some point prior to feeding on the blue honey-baited FTA card.

Results from the one-way ANOVA indicated that the mean numbers of mosquitoes caught by each treatment group were not significantly different, $F(3,60) = 0.901$, $p = 0.466$ ($p > 0.05$). Based on this, simple hay infusion was used for all ensuing field deployments.

4.2. Field results

Throughout the August trapping period, a total of 2,003 mosquitoes were caught (Table 1). Of these, 1,834 (91.6%) were females. Of the females, 96.3% were identified as *Cx. pipiens* s.l./*Cx. torrentium* (n = 1,766, 88%).

In 72 trapping events, the mean number of mosquitoes caught per

Table 1

Mosquito species caught during the August trapping period.

Species	No. collected	
	Male	Female
<i>Cx. pipiens/torrentium</i> complex	163	1766
<i>Cx. hortensis</i>	4	2
<i>Ae. japonicus</i>	0	52
<i>Ae. vexans</i>	0	1
<i>An. maculipennis</i> complex	1	3
<i>An. plumbeus</i>	0	5
<i>Cs. annulata</i>	1	4
<i>Cs. longiareolata</i>	0	1
Total	169	1834

trap was 25, with a minimum of 1 and a maximum catch size of 153 mosquitoes. The mean percentage of mosquitoes already deceased upon collection was 21% with a range from 0% to 100%. On average, 74.4% of mosquitoes had fed on an FTA card upon collection indicated by the presence of blue colouring in their digestive tract (Fig. 3).

Fifteen traps (20%) were positive for USUV during the sampling period. In 11 traps, USUV was detected in the mosquito samples, consisting of 13 positive pools from a total of 177 pools (7%) (Table 2). With 13 positive pools from 1834 total specimens tested, an MIR (minimum infection rate) of 7 was found. Eight of the USUV-positive pools contained unfed mosquitoes and 5 pools contained fed mosquitoes (Table 2). Ten positive pools consisted of *Cx. pipiens* s.l. mosquitoes. The three remaining pools consisted of three *Ae. japonicus*, one *An. maculipennis* s.l., and two *An. plumbeus* (Table 2). Four FTA cards at four different locations were positive across the sampling period (Tables 5 and 6). No traps with USUV-positive mosquitoes had a correspondingly USUV-positive FTA card. All traps with positive FTA cards contained some mosquitoes which had fed on the cards but provided negative mosquito samples. Twelve out of the 18 locations had either a mosquito pool or an FTA card tested positive for USUV during this study.

Temperatures throughout the entire sampling period showed a normal distribution as assessed by a Kolmogorov-Smirnov test ($p = 0.2$). An ANOVA comparing the 48-h temperature recordings for each individual sampling period showed that the means of these periods had significant differences $F(3, 188) = 18.931$, $p = <0.001$. Both the Tukey and Bonferroni post-hoc tests showed that the temperatures from the 48-hour sampling period for the 12th of August were significantly different to the other days as shown by p-values <0.05 , with temperatures from the sampling period for the 12th of August averaging 26.5°. Based on this, temperatures for the four sampling days were reclassified as a categorical variable with average temperatures above or below 26° with a binary yes/no outcome.

The number of mosquitoes caught over the 48-hour trapping period appeared to be slightly higher on the sampling day with the 48-hour average temperature exceeding 26°. An independent samples t-test showed a significant difference in mean number of mosquitoes caught on days where the average 48-hour temperature exceeded 26° compared to days where it was lower than 26° ($t = 2.701$, $p < 0.05$), with the average number of mosquitoes caught higher in the warmer period.

There was a significant difference in mean number of mosquitoes already dead in the trap on days where the average 48-h temperature



Fig. 3. Example of mosquitoes showing blue colouring in the digestive tract as an indicator of having fed on an FTA card. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

USUV positive pools of mosquitoes. Numbers show numbers of individuals in pool. Feeding status showing if mosquitoes had fed from the FTA card shown as Y/N in final column.

Positive pool no.	<i>Cx. pipiens s. 1/torrentium complex</i>	<i>Ae. japonicus</i>	<i>An. maculipennis complex</i>	<i>An. plumbeus</i>	Fed (Y/N)
2	9				N
3	1				N
5	2				N
10	9				N
13	22				Y
22	3				Y
47			1		Y
77				2	N
79		3			Y
94	34				Y
118	5				N
123	2				N
176	1				N

exceeded 26° compared to days where it was lower than 26° ($t = 10.617$, $p < 0.001$), with the average number of mosquitoes already deceased upon collection higher in the warmer period.

Cross tabulation of positive or negative traps against temperature suggested that where the 48-h average temperature exceeded 26°, there may be a lower likelihood of finding a positive trap, however a Fisher's Exact test showed that this difference was not statistically significant ($p = 0.746$).

Despite the mean percentage of mosquitoes fed being slightly higher in catches from positive traps, an independent samples t -test showed that this difference was not statistically significant, $t(70) = 0.575$, $p = 0.567$. There was no statistically significant relationship between the average number of mosquitoes that fed and a positive or negative trap.

Despite no virus positive FTA cards found on days where the 48-hour average temperature exceeded 26°, a Fisher's exact test showed that there was no statistically significant non-random association between temperature and FTA card result ($p = 0.566$).

Furthermore, cross tabulation would suggest that it was more likely to find a positive FTA card at shaded locations, but the Fisher's Exact Test p value of 0.231 exceeded 0.05 so the apparent association could not be considered significant.

5. Discussion

This study represents the first successful instance of the use of FTA cards for the detection of USUV in Germany and confirms previous results from Switzerland (Wipf et al., 2019).

5.1. Infusion attractant

In terms of oviposition substrate, hay infusions can differ in attractiveness for different species and even be repellent for some taxa (Jackson et al., 2005). For *Culex pipiens*, factors like the ingredients used, the preparation method, age of the infusion, weather conditions, as well as if the trap was new or had already been used before affect attractiveness (Lampman and Novak, 1996). Hay infusion has previously been found to be highly attractive to *Culex pipiens* (Jackson et al., 2005) and Lampman and Novak (1996) found that lactalbumin, brewer's yeast, ethanol and other additives were not required to create an attractive infusion. The Latin square results in this study support these findings and showed that even a basic hay infusion is highly attractive to *Culex* mosquitoes in Germany and is no less attractive than more labour or cost intensive variations.

In a study by Jackson et al. (2005) it was noted that hay infusion became more attractive to *Culex* mosquitoes in comparison to a manure-based attractant when it was brewed during periods of higher

temperatures. It was hypothesised that there may be a temperature threshold for microbial activity to reach the levels required for release of attractants. No temperature controls were employed while hay infusion was brewed for this study, though it was brewed outdoors during periods of high temperatures. It is therefore possible that attractiveness could vary for each batch. To overcome this issue, Jackson et al. (2005) suggest leaving the infusion to ferment for a longer period or carry out the process indoors where temperature can be regulated.

5.2. Field application for disease surveillance

Most of the positive pools of mosquitoes consisted of *Culex pipiens*. What differentiates this result from many previous studies is the number of collected mosquitoes and pools tested. A total of 1,834 females were collected and split into 177 pools for analysis, 13 of which were positive for USUV, giving an MIR of 7. Ten of the positive pools contained *Cx. pipiens* but the remaining three positive pools contained small numbers of *Ae. japonicus* ($n = 3$), *An. maculipennis* ($n = 1$), and *An. plumbeus* ($n = 2$). *Ae. japonicus* has previously been found with disseminated infections in the field in Austria (Camp et al., 2019) and under laboratory conditions (Abbo ID et al., 2020), suggesting they may play a minor role in transmission. Widespread infection of the species has to date not been found, nor has successful transmission of the virus to a host been observed. No published accounts of detections of USUV in either *An. maculipennis* or *An. plumbeus* were able to be found although the former has previously been detected with WNV (Kemenesi et al., 2014). However, neither species is thought to play a major role in USUV transmission, with both species considered zoophilic (Börstler et al., 2016; Brugman et al., 2015; Jöst et al., 2010). Importantly, these this study only used gravid traps and as such did not collect the vast numbers of host-seeking mosquitoes as is typical when using CO₂ baited traps (Healy et al., 2015). Although CO₂ baited traps have been found to significantly outperform gravid traps in terms of absolute numbers caught and are often considered the most suitable for flavivirus surveillance (L'Ambert et al., 2012; Williams and Gingrich, 2007), this trap type does not specifically target gravid female mosquitoes which are more likely to be infected by viruses (L'Ambert et al., 2012; Cummings, 1992). Samples from gravid traps have previously been found to have an infection rate up to 33 times higher than that of standard light traps with 2.29 versus 0.07 infected *Culex* mosquitoes per 1000 caught (Williams and Gingrich, 2007). Such figures highlight the benefit of using box gravid traps as a much lower trapping effort is required to obtain a suitable sample.

5.3. Influence of temperature on trap performance

Six out of 15 of the positive pools of mosquitoes from the USUV sampling were found in the week following the hottest recorded temperatures for the sampling period. However, there was no similar difference in the number of positive FTA cards. On the other hand, no positive FTA cards were found during the hottest trapping period. The limited sampling period and number of traps deployed were insufficient to gather enough data to identify relationships between potential time lags between high temperatures and a virus positive collection, but it is an interesting observation nonetheless and warrants further investigation.

Although more mosquitoes were caught where the 48-hour average temperature exceeded 26 °C ($t = 2.701$, $p < 0.05$), more mosquitoes were also already deceased upon collection ($t = 10.617$, $p < 0.001$). This could have had implications for the likelihood of a pool of mosquitoes testing positive for virus where environmental factors including temperature, humidity, fungi and bacteria may begin to degrade RNA (Hall-Mendelin et al., 2010; van den Hurk et al., 2012; Johansen et al., 2002) although this study was unable to directly evidence this relationship.

The lack of a correspondingly positive pool of mosquitoes for those

cards which were positive in the USUV survey is likely explained by degradation of the RNA in the mosquito sample. Regardless of the status of the FTA card, there were multiple incidences where only individual mosquito specimens from a trap tested positive for virus. This finding arose incidentally through the way the testing pools were split firstly by species, and then by feeding status. It is likely that even in larger pools there were only individual infected mosquitoes. If this mosquito was already dead before collection and had lain in a hot trap, this information could have quickly been lost. Additional opportunities for RNA degradation may exist once the trap has been collected, during processing and identification of the sample.

Only four positive FTA cards were found in this study, with the temperature the cards were exposed to likely a key explanatory factor of this. There is an overall lack of information regarding the conditions under which FTA cards can be successfully used to isolate RNA (Cardona-Ospina et al., 2019). There is some evidence that FTA cards with their sample can be stored for at least one month at temperatures up to 37 °C. However, a study using dengue positive blood samples found significantly reduced recovery of RNA from FTA cards, especially when stored at 37° (Dauner et al., 2015). No data could be found for temperatures above this, nor for how the temperature at time of transfer may affect the successful transfer of genetic material to the FTA card.

The outdoor maximum temperatures exceeded 30 °C on two of the trapping days and 35 °C on the remaining days. Crucially however, the outdoor temperature will have been lower than the maximum temperatures experienced inside the traps themselves. The black casing of the trap will mean that the internal temperatures will likely have been significantly higher than outside. In a study on the effects of sunlight on temperatures within black polyethylene plant containers, soil temperatures reached almost 50° 1 cm from the side of the container on a day where the maximum outdoor temperature did not exceed 25 °C (Young and Hammitt, 1980). An increase of 20 °C would therefore not be inconceivable and could result in the internal trap temperature reaching over 65 °C. With the fan remaining on during trapping, it is likely that this would have somewhat moderated the internal trap temperature, though it would not have completely countered the effects of solar heating. The use of dataloggers in the traps would be a useful step to determine the temperature extremes the cards were exposed to.

Although not statistically significant, it is noteworthy that the only traps where positive FTA cards were found, were traps which were positioned in full shade. Additionally, there was a much higher ratio of positive FTA cards found during sampling for WNV in Germany when outdoor temperatures were cooler (Fynmore et al., 2021). An examination of the effect of such high temperatures on successful virus transfer and preservation on the FTA card is warranted in further studies. Despite the low number of positive FTA cards found, the fact that these cards were positive despite the mosquito pool testing negative shows that the cards may still be a sensitive virus detection method, but only where conditions are favourable for their use.

5.4. Mosquito feeding and FTA card performance

On average, 74.4% of the trapped mosquito females had fed on an FTA card. This figure matches the findings by Wipf et al. (2019) who reported the same average feeding rate across samples caught in box gravid traps. It remains possible however, that feeding rates were lower than what may have been achieved had the fan periodically been turned off. Such a hypothesis is supported by evidence of feeding rates of over 90% in passive traps (Ritchie et al., 2013). The addition of features such as a closing vent on a timer could go some way to eliminate this issue but would add additional fabrication costs and complexity to the design. The value of such modifications could be assessed in a comparison of feeding rates in box gravid traps with and without fan control but was beyond the scope and resources of this study.

It is noteworthy that only five of the 13 positive pools had fed on the FTA cards (Table 2) despite most specimens captured showing signs of

feeding. It is hypothesised that this may be an artefact of the temperatures experienced in the field and the time of collection, with mosquitoes which had been captured towards the end of a 48-h trapping period not having time to feed before being euthanised, whilst mosquitoes which had been trapped and fed earlier more likely to have been exposed to high temperatures before collection.

None of the USUV positive pools of mosquitoes had a correspondingly positive FTA card. The number of positive pools also exceeded the number of positive FTA cards in a ratio of over 3:1. Both, Flies et al. (2015) and Wipf et al. (2019), put forward testing the mosquitoes captured in a collection chamber for virus as a form of control or to determine the vector involved where an FTA card is positive, however this study shows that it cannot be assumed that a negative FTA card will mean a negative pool of mosquitoes, nor that a negative pool of mosquitoes will correspond to a negative FTA card. It should be noted that not all infected mosquitoes will be infectious. For example, a low dose of virus ingested by the mosquito may lead to a failure to disseminate to secondary tissues such as the salivary glands (Anderson et al., 2010). In such cases, a pool of mosquitoes may test positive for virus although the mosquito presents no transmission risk and is unable to transmit virus to an FTA card.

Curiously, laboratory testing by Hall-Mendelin et al. (2010), showed that virus could be detected on the FTA cards even when no blue colouring could be seen in the mosquito. This could be due to saliva being transferred to the card by probing but the mosquito not continuing feeding (Styer et al., 2006). Furthermore, in field trials by the same authors, virus was detected on FTA cards several times even in the absence of a positive mosquito sample and positive mosquitoes were periodically found in traps without a positive FTA card. Both are situations which also arose frequently in this study.

Additional factors which may play a role in reduced RNA recovery from the FTA cards may be related to insufficient elution, due either to the amount of the sample on the card, the elution method, or the way the RNA was stabilised on the card itself (Dauner et al., 2015). Importantly, it is hypothesised that where viral titres are low, this may lead to inaccurate results from FTA cards (Dauner et al., 2015). For example, when offered a feed through an artificial membrane, mosquitoes spend less time probing before feeding (Ribeiro et al., 1984) and the amount of virus injected whilst probing is lower than from those that have fed (Styer et al., 2006). It is therefore possible that some of the FTA cards which were negative for flaviviruses were caused by too little viral RNA or degradation of the limited RNA present. The latter is also true for samples where mosquitoes had died before being euthanised and frozen. The number of positive FTA cards and positive pools of mosquitoes is therefore the lowest possible number detectable, as false positives are extremely unlikely.

The simplified trapping and surveillance method provided by the FTA card system with a faster time to results from trapping lends itself well to use as an early warning system and could be easily scaled up for a larger surveillance programme (Hall-Mendelin et al., 2010; Fynmore et al., 2021). However, this study and others make it clear that more work needs to be done to establish the temperature and humidity boundaries that such collection methods can withstand (Dauner et al., 2015). This knowledge would be of particular significance to a study such as this where the FTA cards are kept moist for the duration of the time in the field and are exposed to extremes in temperature.

6. Conclusion

This study supports the conclusion that Reiter/Cummings type box gravid traps constitute a suitable trap for the surveillance of arboviruses such as USUV in populations of *Culex* mosquitoes. Despite relatively low numbers of mosquitoes being caught overall, gravid females, the most appropriate subpopulation for viral testing, constituted a much larger proportion of the sample. Both the traps and water-based hay infusion attractant are simple to create from low-cost, readily available materials.

This makes them a very cost-effective option. The trap durability and internal collection chamber serve to protect the sample from many of the factors which would otherwise lead to a damaged sample. The ability to incorporate an FTA card makes it an attractive option where mosquito processing is unwanted or prohibitively expensive. However, both the mosquito sample and particularly the FTA card may be vulnerable to heat damage. FTA cards may be beneficial in terms of returning positive results only in the presence of infectious rather than infected mosquitoes and may under certain circumstances better retain RNA for analysis than the mosquito sample. However, the conditions under which an FTA card continues to return a reliable result, especially during periods of extreme heat, remain unknown and require investigation. Likewise, the abiotic conditions affecting sample size and quality including air flow and internal trap temperature should be researched.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

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CRedit authorship contribution statement

Noelle Fynmore: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Investigation. **Renke Lühken:** Writing – original draft, Investigation. **Konstantin Kliemke:** Investigation. **Unchana Lange:** Investigation. **Jonas Schmidt-Chanasit:** Writing – review & editing. **Peter W.W. Lurz:** Writing – review & editing. **Norbert Becker:** Conceptualization, Methodology, Writing – original draft, Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

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Supplementary materials

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