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Evaluation of a temperate climate mosquito, *Ochlerotatus (Aedes) detritus,* as a potential vector for Japanese encephalitis virus

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**Abstract**

Great Britain has not yet experienced a confirmed outbreak of mosquito-borne virus transmission to people or livestock despite numerous autochthonous epizootic and human outbreaks of mosquito-borne diseases in the European mainland. Indeed, it has not been established if British mosquitoes are competent to transmit arboviruses. Therefore, we assessed the competence of a local (temperate) British mosquito species, *Ochlerotatus (Aedes) detritus,* for a member of the Flavivirus genus, Japanese encephalitis virus (JEV) as a model for mosquito-borne virus transmission. We also evaluated JEV competencein a laboratory strain of *Culex quinquefasciatus*,an incriminated JEV vector, as a positive control. *O. detritus* adults were reared from field-collected juvenile stages. In oral infection bioassays, adult females developed disseminated infections and were able to transmit virus as determined by isolation of virus in saliva secretions. When pooled from 7 to 21 days post infection, 13 and 25% of *O. detritus* were able to transmit JEV when held at 23 and 28°C, respectively. Similar results were obtained for *C. quinquefasciatus*. To our knowledge, this study is the first to demonstrate that a British mosquito species, *O. detritus,* is a potential vector of an exotic flavivirus.

**Introduction**

The emergence of mosquito-borne viruses in subtropical and temperate regions of Europe ([Phipps *et al.*, 2008](#_ENREF_33)) in recent years has raised concerns about the risk of an outbreak occurring in Great Britain (GB). However, the risk to GB from mosquito-borne arboviruses is unknown. A major knowledge gap is the vector competence of GB’s indigenous mosquitoes for arboviruses. While there have been no reports of outbreaks of disease caused by mosquito-borne viruses, studies in GB have reported the serological detection of antibodies to West Nile virus (WNV), Usutu virus and Sindbis virus in both migrant and non-migrant wild bird species ([Buckley *et al.*, 2003](#_ENREF_4)), and to WNV in sentinel chickens raised on a farm ([Buckley *et al.*, 2006](#_ENREF_3)), suggesting that some transmission of arboviruses may occur.

Vector competence is a measure of the ability of a mosquito to become infected with, allow replication of, and transmit virus to a susceptible host ([Kramer & Ebel, 2003](#_ENREF_20)). The extrinsic incubation period (EIP) is an important aspect of the biological transmission of a pathogen in a vector. EIP is the period from ingestion of the pathogen to the point where onward transmission is possible ([Higgs & Beaty, 2005](#_ENREF_15)). EIP determines at what point the vector will be able to transmit infectious virus. The duration of EIP varies with temperature, with the general trend of higher temperature leading to faster pathogen replication and dissemination and hence shorter EIP duration.

At present, there are thirty-four species of mosquitoes recorded in the British Isles comprising six species of Anophelinae (genus *Anopheles*) and 28 species of Culicinae in seven genera: *Aedes* (3), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana (1), Ochlerotatus* (11) and *Orthopodomyia* (1) ([Medlock & Vaux, 2009](#_ENREF_27)). With the exception of the recently (re)discovered *Culex modestus* ([Marshall, 1945](#_ENREF_23); [Golding *et al.*, 2012](#_ENREF_13); [Medlock & Vaux, 2012](#_ENREF_26)), all of these mosquitoes are thought to be native species. However, to our knowledge there is no information on the vector competence of these resident British populations to any arbovirus.

*Ochlerotatus detritus* Haliday 1833 (Diptera: Culicidea) was selected in this study as a model to determine the vector competence of a temperate mosquito originating from GB. Because of its relative abundance in our sampling site (Cheshire county, GB), accessibility and biting behaviour, it was found to be ideal for vector competence evaluation at the time this study was implemented. It is one out of thirteen British species of mosquito that can be considered a potential bridge vector should any mosquito-borne virus emerge in the UK ([Medlock *et al.*, 2005](#_ENREF_25)). *O. detritus* has been shown to feed on both birds and humans ([Service, 1971](#_ENREF_39)) and therefore can potentially transmit flaviviruses to humans from their natural cycle in birds. It is a salt marsh mosquito found in the low-lying coastal and some inland saline waters ([Rees & Snow, 1996](#_ENREF_36)). Though *O. detritus* has a widespread but patchy distribution in GB ([Snow *et al.*, 1998](#_ENREF_41); [Medlock *et al.*, 2005](#_ENREF_25)), in coastal areas where it is found, this mosquito causes the greatest human biting nuisance of any British mosquito ([Clarkson & Setzkorn, 2011](#_ENREF_8)). *O. detritus* oviposits in salty ground prone to periodic flooding and usually a generation follows each immersion ([Snow, 1990](#_ENREF_40)), hence it is multi-voltine. *O. detritus* bites humans persistently with adults appearing from March to November and overwinters as 4th instar larvae. Biting occurs mainly outdoors ([Service, 1971](#_ENREF_39)). *O. detritus* is distributed throughout European coastal districts from the Baltic to the Aegean, Mediterranean and Red sea, and in inland saline areas in Europe and North Africa ([Cranston *et al.*, 1987](#_ENREF_10)). Another study also mentions the presence of *O. detritus* in the Qinghai-Tibet Plateau, China ([Li *et al.*, 2010](#_ENREF_21)).

We used Japanese encephalitis (JE) virus as the model virus to evaluate vector competence in *O. detritus*. JE virus (JEV) has been considered one of ten important zoonotic pathogen threats, capable of spreading to new regions ([Johnson *et al.*, 2012](#_ENREF_17); [Kilpatrick & Randolph, 2012](#_ENREF_19)). JEV is also the prototype of a sero-complex of closely related flaviviruses, which includes WNV and Usutu virus (USUV). JEV is an arbovirus that is maintained in a zoonotic cycle, which can be both enzootic and epizootic. This cycle involves pigs as the major reservoir/amplifying host, water birds as carriers and mosquitoes (in particular *Culex tritaeniorhynchus*) as vectors. Humans are considered dead-end hosts because they produce low viraemia levels over a limited time-frame that are insufficient to infect feeding mosquitoes ([Scherer *et al.*, 1959](#_ENREF_38); [Chan & Loh, 1966](#_ENREF_6); [Impoinvil *et al.*, 2013](#_ENREF_16)). The disease caused by JEV has an estimated worldwide annual incidence of 70,000 human cases with approximately three quarters occurring in children aged 0 to 14 years ([Campbell *et al.*, 2011](#_ENREF_5)). Roughly, one quarter of encephalitis patients will die while about one half of the survivors will develop permanent neurologic and/or psychiatric impairment ([Unni *et al.*, 2011](#_ENREF_45)). Although commercial inactivated vaccines are available against JEV, it still remains the most important member of the JEV sero-complex and the most widespread of a group of antigenically related mosquito-borne viruses that cause encephalitis in man.

We investigate for the first time, the vector competence of a British mosquito species *O. detritus* for JEV at different temperature. We address the following research questions: 1) *O. detritus* will be susceptible to infection with JEV: 1) *O. detritus* will be susceptible to infection with JEV; 2) If *O.detritus* is capable of transmitting JEV, it will be more competent at the higher temperature of 28°C than at the lower temperature of 23°C.

**Materials and Methods**

Mosquitoes

Mosquitoes used in this study were derived from wild-caught larvae of *O. detritus* sourced locally and *Culex quinquefasciatus,* Say (Recife strain), a colonized mosquito from Brazil maintained at the Liverpool School of Tropical Medicine. *C. quinquefasciatus* was used for validation since JEV has been isolated from this mosquito previously ([Weng *et al.*, 1999](#_ENREF_48); [Halstead & Tsai, 2004](#_ENREF_14); [Nitatpattana *et al.*, 2005](#_ENREF_31); [Changbunjong *et al.*, 2013](#_ENREF_7)) and found to be competent for JEV in infection studies ([Mourya *et al.*, 2002](#_ENREF_29); [van den Hurk *et al.*, 2003](#_ENREF_46); [Liu *et al.*, 2012](#_ENREF_22)).

*O. detritus* immatures (larvae and pupae) were collected from pools on Quayside saline marsh in northwest England (GPS coordinates: 53.277073N, -3.067728W) and transported to the Liverpool School of Tropical Medicine (LSTM) insectary. They were reared in trays (15 × 30 × 5 cm) in the same water from which they were collected. Identification of fourth instar larvae was carried out using the identification keys for British mosquitoes ([Cranston *et al.*, 1987](#_ENREF_10)). A colony for this mosquito was not established because laid eggs failed to hatch; hence immatures were collected fresh for every experiment. *C. quinquefaciatus* were obtained from a colony maintained in the LSTM insectary. Larvae were hatched, then divided among 15 × 30 × 5 cm trays with approximately one litre of de-chlorinated water and fed on brewer’s yeast tablets (Holland & Barrett, Nuneaton, Warwickshire, UK) as needed. Approximately, 150 to 200 3rd to 4th instar larvae were reared in each pan for *O. detritus* and *C. quinquefasciatus.* Once the larvae started pupating, pupae for both mosquito species were harvested daily and transferred to separate BugDorm cages® (BioQuip, Rancho Dominguez, CA) (30 × 30 × 30 cm) where they would emerge as adults. All adults and larvae were maintained at 27°C with a relative humidity of 80% and 12:12 light: dark cycle. The adults were provided with 10% sucrose and water *ad libitum.*

Cells and viruses

The Muar strain of JEV was used in all infection experiments. We used this virus strain because it has been fully sequenced and characterized by our laboratory ([Mohammed *et al.*, 2011](#_ENREF_28)). Vero cells were maintained in Dulbecco Modified Eagle’s Minimal Essential Medium (DMEM) Sigma-Aldrich) media containing 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-glutamine and 50 µg/ml Penicillin/Streptomycin.

Vector Competence

Field populations of *O. detritus* F0 mosquitoes and *C. quinquefasciatus* colony mosquitoes were tested for JEV vector competence at two temperatures (23°C or 28°C) and at time points 0, 1, 3, 7, 14, and 21 days post-infection (dpi; i.e. after offering an infectious blood-meal). Time point 0 represent mosquitoes collected 1-hour after offering an infectious blood meal.

Mosquitoes were held at 23°C or 28°C in a Sanyo incubator model MIR-153 with a photoperiod of 12:12 light: dark cycle. A pan of water was kept in the incubator to maintain a relative humidity range of 70 – 90% relative humidity.

Mosquitoes were sampled at 0, 1, 3, 7, 14, and 21 dpi at both 23°C and 28°C. The two temperatures were used to provide preliminary evidence for any important effects of temperature on the level of vector competence of *O. detritus*.

*Per oral infection and transmission assay*

 All work with infectious blood meals was undertaken in the Arthropod-containment level-3 (Ar-CL3) facilities at LSTM. Viral stocks were diluted prior to infecting mosquitoes to ensure the final titre was correct. Infectious blood meal containing virus from frozen stock was prepared by combining defibrinated horse blood (Thermo Oxoid Remel), with the appropriate volume of virus stock and 100 µl of adenosine 5’-triphosphate (ATP 0.02 µm) as a phagostimulant to a final concentration of 6 logs pfu/ml

Seven day-old adult female mosquitoes were aspirated from their cages into round 0.5 litre polypropylene plastic containers. Fine nylon netting was placed over the mouth of the container to provide ventilation and prevent the escape of the mosquitoes. The netting was secured by rubber bands and the hollowed-out lid of the container. A small slit was made in the net in order to fit the mouth aspirator. The slit was closed with cotton wool. The mosquitoes were deprived of sucrose solution and maintained on water soaked cotton balls for 24 hours prior to blood feeding. We attempted to feed approximately one hundred mosquitoes for each experiment in order to achieve a minimum of 50 mosquitoes for assessment of infection.

Peroral infection was achieved by exposing mosquitoes to a suspension of defibrinated horse blood and the Muar strain of JEV, using a Hemotek membrane feeding system (Hemotek limited Accrington, Lancashire, UK) for 1 hr at ~23°C (50 – 70% humidity) in the dark. Parafilm® M was used as the membrane. In all cases 0.5 ml aliquots of the infectious blood meal were collected both before and after the mosquitoes were fed and stored at -80**°**C for subsequent virus isolation. This was done to confirm that the virus was viable before and after the blood feed, and determine if there was any change in the virus concentration.

Engorged mosquitoes were chilled and sorted on ice and placed in fresh round 0.5 litre polypropylene plastic containers with fine nylon netting. Fed females were maintained on cotton balls soaked with 10% sucrose solution. Excess sugar solution was squeezed out from the cotton ball to prevent it from dripping into the plastic cups. Cotton balls were changed daily.

Mosquitoes were sampled at 0, 1, 3, 7, 14, and 21 dpi at both 23°C and 28°C. For mosquitoes sampled at 0, 1 and 3 dpi, the whole mosquito body were frozen individually at -80°C in 1.5 ml skirted conical microcentrifuge tubes with external thread O-ring screw-cap containing diluent media (Minimum essential medium (MEM), containing 1% Bovine serum albumin (BSA), 50 µg/ml penicillin/streptomycin, 0.3% Sodium bicarbonate and 2.5 µg/ml Fungizone). Early time points (0 to 3 dpi), representing the eclipse phase of virus production in a mosquito, were sampled to ensure that virus detection reported for later time points (7 to 21 dpi) was the result of new virus production rather than carry over from input virus. The eclipse phase is the period after the ingestion of an infectious bloodmeal by a mosquito where the virus titre decreases to minimal or non-detectable levels which are reached at about 3 to 4 days depending on temperature, virus or vector ([Higgs & Beaty, 2005](#_ENREF_15)). After multiplying in the midgut cells and spreading to other organs including the salivary glands, the virus can then be detected usually from about 7 days after feeding. For mosquitoes sampled at 7, 14 and 21 dpi, saliva was collected from live mosquitoes and mosquito legs were dissected prior to freezing. Each individual sample of these mosquitoes (saliva, dissected legs and the remaining mosquito body) were also placed in a 1.5 ml tube with diluent media and then frozen at -80°C as mentioned above. Manipulation of the mosquitoes was achieved by anesthetizing them using Triethylamine (TEA) FlyNap® (Blades Biological Limited, UK).

Salivary secretions were collected using a modified *in vitro* capillary transmission assay ([Aitken, 1977](#_ENREF_1)). Mosquito mouth parts were inserted into a plastic Micro-Hematocrit capillary tube, (Drummond ®, Cole-Parmer, UK) containing approximately 10 µl of a mixture of virus diluent, 50% sucrose and adenosine 5’-triphosphate (ATP, 0.02 µM) for 30 to 45 minutes. One μl of 1% pilocarpine (Alfa Aesar, Ward Hill, MA, USA) solution in phosphate buffered saline (PBS) and 0.1% Tween 80 was applied to the thorax to stimulate salivation ([Boorman, 1987](#_ENREF_2); [Dubrulle *et al.*, 2009](#_ENREF_12)). Active movement of the maxillary palpi and the stylets observed under a stereoscopic microscope, bubble formation in the media and engorgement of the mosquito were interpreted as a sign of salivation. The contents were then released under pressure into a tube containing 0.5 ml of virus diluent.

Infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body but not in its legs, the mosquito was considered to have a non-disseminated infection. If virus was recovered from both the legs and the body suspension the mosquito was considered to have a disseminated infection and if virus was recovered from its saliva the mosquito was considered to have a transmissible infection ([Turell *et al.*, 1984](#_ENREF_43)).

We defined the infection, dissemination and transmission rates as the number of mosquitoes testing positive for virus in their bodies, legs and saliva, respectively divided by the total number of mosquitoes tested, times 100. We also considered *transition* efficiency – the proportion of infected mosquitoes that have a disseminated or transmissible infection, or the proportion with disseminated infections that have a transmissible infection.

Plaque assay

Body and leg samples were prepared for virus titration by homogenizing using a Disruptor genie® cell disruptor (Scientific Industries, USA) for 5 minutes in a 1.5 ml tube containing 0.5 ml diluent media and two 6mm glass beads (Merck KGaA, Germany). Plaque assays were performed by inoculating 100 µl of the salivary secretions or the supernatant of the homogenized bodies and legs onto a confluent monolayer of Vero cells on a 6-well plate (Costar®, Corning Life Sciences). The plates were then incubated at 37°C at an atmosphere of 5% CO2 for 30 - 60 minutes with rocking every 10 minutes to allow the virus to enter the cells. A 4 ml overlay of MEM, 4% FBS, 50 µg/ml gentamycine, 0.5% Sodium bicarbonate and 2.5 µg/ml Fungizone (amphotecerine B) to limit contamination and 1% SeaPlaque low melting point agarose was then added to the wells and the plates were incubated at 37°C in 5% CO2. After 5 days of incubation, 2 ml of 10% neutral buffered formalin solution was added to each well and the plates left for at least 3 hours with the fixative to ensure complete inactivation of the virus. In order to visualize the plaques the wells were stained with 0.5 ml of crystal violet solution. Samples were scored as virus-positive or virus negative based on the presence or absence of plaques. Viraemia of mosquito carcases was determined for a small subset of mosquitoes (i.e. ~3 mosquitoes per species and temperature for 0, 1, 3, 7, 14 and 21 days). For days 7, 14 and 21, all JEV-positive mosquito body samples with JEV-positive saliva or disseminated infection (i.e. legs positive for virus) were further assessed for their viraemia. Viraemia was also determined for a subset of saliva samples.

**Statistical analysis**

Fisher Exact Test was used to determine if there were significant differences (*P* < 0.05) in rates of infection, dissemination and transmission between temperatures. This was done both at each time point (dpi) and also in pooled analysis (7 to 21 dpi) to overcome issues of small sample size. SISA, an open access online statistics calculator (<http://www.quantitativeskills.com/sisa/>) was used to conduct Fisher Exact Test. Confidence intervals of proportions were calculated using VassarStats (<http://vassarstats.net/prop1.html>). VassarStats uses the Wilson Score Interval method which is more robust when dealing with small number of trials and/or an extreme probability ([Newcombe, 1998](#_ENREF_30)). Sample size in each group was dictated by the feeding success and the survival rates through the days post infection (dpi).

**Results**

There was a constant attrition of mosquitoes during the course of the study. A total of 873 field-collected *O. detritus* were offered an infectious bloodmeal and only 397 (45%) of them engorged, while 506 out of 695 (73%) of the colony mosquito *C. quinquefasciatus* acquired a bloodmeal. Of those that acquired an infectious bloodmeal, more than half of the *O. detritus* died (224 of 397), while about a quarter (150 of 506) of the *C. quinquefasciatus* died during the course of the experiment (Table 1). Mortality of *O. detritus* was especially high (65%) at 28°C. The rate of engorgement in *C. quinquefasciatus* was higher than *O. detritus*.

Though freshly harvested viral stocks, prior to being frozen, were originally estimated to be ~6 logs PFU per ml, assessment of the infectious bloodmeal before and after being placed in the Hemotek artificial feeding system always yielded ~4 logs PFU/ml.

Both mosquito species displayed a typical eclipse phase following oral infection in which the virus titre and detection decreased from 0 to 3 dpi followed by an increase in virus titre and detection from 7 to 21 dpi (Figure 1 and Table 2). This is attributable to the reduction in virus titre after day 0 as ingested virus either infects cells or gets digested; virus successful in infecting cells replicates to detectable levels several days later. Viraemia of saliva samples ranged from ~1 log to ~ 3 logs PFU/ml for both mosquitoes at both temperatures.

Both mosquito species were susceptible to JEV infection with infection rates ranging from 32 to 100% for *O. detritus* and 25 to 100% for *C. quinquefasciatus* (Table 2). In general, higher infection, dissemination and transmission rates were reached at later time points although there was some variation. Dissemination rates of both species tended to be similar to the infection rates. Transmission rates tended to be lower than dissemination rates. Nevertheless, 33 – 67% of *O. detritus*, and 50 – 70% of *C. quinquefasciatus*, had developed transmissible infections by 21 dpi.

Overall, when 7 to 21 dpi are combined, the field populations of *O. detritus* were competent for JEV with 62% infection, 54% dissemination and 13% transmission rate at 23°C and 62% infection, 58% dissemination and 25% transmission rate at 28°C. The rate of infection, dissemination and transmission in *O. detritus* did not differ significantly at the two temperatures in individual or pooled analysis. For *C. quinquefasciatus*, when 7 to 21 dpi are combined, JEV competence rates were 50% infection, 35% dissemination and 15% transmission rate at 23°C and 61% infection, 45% dissemination and 29% transmission rate at 28°C. In addition, for *C. quinquefasciatus*, when the analysis was done individually for each time point (7, 14, and 21 dpi), there were marginal (0.05 < p <0.1) or non-significant effects of temperature on the rate of infection, dissemination and transmission. However, sample sizes for individual time points are small. When the data for later time points (7 to 21 dpi) are pooled, the effect of temperature on the transmission rate was significant (χ2 = 7.199, df = 1, p = 0.014).

To describe the transition efficiency of the virus after overcoming the midgut barrier in the mosquito, the number of mosquitoes with a disseminated infection out of the number infected (dissemination efficiency) was examined and the number of mosquitoes able to transmit out of those disseminated (transmission efficiency) was also examined at day 7, 14, and 21 dpi (Table 3). Both mosquitoes attained 100% dissemination efficiency by 21 dpi. However, transmission efficiency was variable with the rates always higher at 21 dpi.

**Discussion**

This is the first study to investigate the biological competence of a mosquito of British origin (*O. detritus*) to an arthropod-borne virus. *O. detritus* was susceptible to laboratory infection with JEV at 23°C and 28°C, with virus detectable in the saliva of some individuals as early as 7 dpi, and it therefore appears to be a competent vector for this flavivirus.

Since the *O. detritus* mosquito population used in this studyis a temperate variety, it showed poor survival when incubated at 28 °C, and there was high mortality during the experiments; hence, no mosquitoes survived greater than 21 dpi. This mosquito was also not adapted to acquiring a blood meal from an artificial feeder and that may have led to lower numbers of mosquitoes acquiring an infectious blood meal.

In our study, the transmission rate for *O. detritus* was only 19% when averaged for the two temperatures at the different days post infection. However, it is important to note that the medium used to collect the saliva can affect the amount of virus detected; because we used an aqueous solution in the capillary tube assay we may have underestimated the amount of virus being secreted by the mosquito ([Colton *et al.*, 2005](#_ENREF_9); [Turell *et al.*, 2006](#_ENREF_44)). While animal infections with the mosquito would have been a better model to confirm transmissibility, we did not have the facilities to do this. In our study, salivary viraemia ranging from 1 log to 3 logs PFU/ml were produced. The viraemia produced in the saliva secretion of both mosquito species is likely to cause infections in susceptible birds, humans or other mammals.

The decrease in detectable titres of JEV in *O. detritus* and *C. quinquefasciatus* during the first 3-days after an infectious blood meal indicated an eclipse phase in virus replication. At 23°C, for both species, the infection rates are very similar at 3 and 7 days (33 vs. 32%; 27 vs. 25%). We cannot unequivocally prove that the entire virus population at the earlier time point is residual or that the entire virus population at the later time point is newly replicated. However, since we detected viral dissemination and transmission by 7 dpi, it is likely viral replication has occurred by then and this is not “carry-over” input virus; the increase in viral titre between 3 and 7 dpi suggests the same. Early in the eclipse phase, the rate of reduction in virus titre and detection appears to have been sharper at 28 compared to 23°C.

It remains to be seen whether *O. detritus* is competent at normal GB temperatures. We found no significant difference in the infection, dissemination and transmission rates in *O. detritus* at 23°C and 28°C. This was unexpected as studies have shown that increases in temperature often reduce the EIP, therefore increasing infection, dissemination and transmission rates ([Davis, 1932](#_ENREF_11); [Takahashi, 1976](#_ENREF_42); [Kay *et al.*, 1989](#_ENREF_18)). For *O. detritus,* since only 24 mosquitoes were assessed at 28°C while a total of 63 mosquitoes were assessed at 23°C, it is possible that our results may have been affected by small sample sizes, limiting the power of the study to detect a difference. It should also be noted that an increase in temperature could also reduce the adult lifespan of mosquitoes and this may interrupt transmission (see Table 1). In contrast, the pooled results for *C. quinquefasciatus* at the two temperatures were significantly different.

JEV disseminated well in the bodies of both mosquito species, as demonstrated by high dissemination rates (i.e. virus found in the legs). This may indicate that JEV was able to overcome the midgut barriers in the mosquitoes. However, transmission rates were lower than dissemination rates (see Table 3). While, these results are consistent with the existence of a salivary gland infection barrier, but further work, with larger sample sizes, is needed to confirm this.

Our result of 19% transmission rate by *O. detritus* must be gauged against the vectorial capacity indicators to determine the likelihood of sustained transmission of JEV for this vector. Early studies in Britain have estimated the feeding rate of *O. detritus* on birds to be 3.7% (3-bird blood positives of 81-decernible tests), while the feeding rate on humans was 33.3% (27 of 81) ([Service, 1971](#_ENREF_39)). Feeding behaviour on other mammals are 1.2 % (1 of 81) for pigs and 49.4% (40 of 81) for bovids. Despite the low feeding rate on JEV amplifiers (i.e. birds and pigs), there is still a sizeable population of the *O. detritus* in Cheshire County ([Clarkson & Setzkorn, 2011](#_ENREF_8); [Medlock *et al.*, 2012](#_ENREF_24)), which may make it possible for transmission to be sustained by this vector. Nonetheless, other factors to be considered are survival of mosquitoes at optimal conditions.

As mentioned earlier, *O. detritus* and JEV were selected primarily out of convenience. However, *O. detritus* is a relevant mosquito to study as it has high human biting rates ([Clarkson & Setzkorn, 2011](#_ENREF_8)), and is considered a potential bridge vector for arboviruses such as WNV ([Medlock *et al.*, 2005](#_ENREF_25); [Osorio *et al.*, 2012](#_ENREF_32)). JEV, recognised as a virus with the potential to expand in range ([van den Hurk *et al.*, 2009](#_ENREF_47)), is also a relevant model to use; this is underscored by the recent detection of the JEV gene sequence in a pool of *C. pipiens* in Italy. In 2010, the detection of the NS5 gene RNA sequence of the JEV was reported from one pool of *C. pipiens* mosquitoes collected in north-eastern Italy ([Ravanini *et al.*, 2012](#_ENREF_35)). This report suggested that the threat of the introduction of arboviral diseases of tropical origin to temperate regions is ever present and requires constant vigilance ([Platonov *et al.*, 2012](#_ENREF_34)).

In the case of JEV, suitable vertebrate hosts for virus amplification are pigs and water-birds. The marsh where we sourced *O. detritus* is a protected conservation area that is frequented by several avian species including water birds such as the little egret and different varieties of ducks and geese and other aquatic avian. However, the susceptibility of British birds to JEV is not known.

Humans are considered dead-end hosts in the transmission of JEV and therefore its introduction in the UK would most likely be through transportation of infected mosquitoes on planes, ships or cars; trade in domestic animals and also infected migratory birds which may play a critical role in the long distance transportation of the virus ([Platonov *et al.*, 2012](#_ENREF_34)). Our demonstration of the presence of a competent local vector highlights the need for continued vigilance to prevent local transmission of arboviruses in the UK and suggests that mosquito control will form part of the intervention strategy in the event of disease emergence.

Some of the limitations of the study include the following: I) We used relatively high temperatures (i.e. 23 and 28°C) which were beyond the average summer range temperature experienced in Cheshire where the *O. detritus* were sourced. For example, July is the warmest month, with mean daily maximum temperatures approaching 21°C in Cheshire (<http://www.metoffice.gov.uk/climate/uk/nw/print.html>). The higher temperature certainly impacted mosquito survival; still it is not clear to what extent it played a role on the overall JEV susceptibility. There were no significant differences between *O. detritus* kept at 23 and 28°C but this may be due to sample size. Other studies have demonstrated transmission of JEV in mosquitoes held at 20°C ([Takahashi, 1976](#_ENREF_42)). II) Though we started with relatively large numbers of mosquitoes, our sample size was relatively low at the later time points (i.e. 14 and 21 dpi). The difficulty of consistently getting mosquitoes from the field and keeping them alive long enough in the laboratory for assessment was a challenge. Future study should focus on holding mosquitoes at more optimum survival conditions and doing more replicates to get larger sample sizes at later time points. III) We did not use freshly harvested virus for our infections. Rather, we used frozen stocks out of convenience and convention. This may have affected the infection efficiency as suggested in other studies ([Richards *et al.*, 2007](#_ENREF_37)). Nonetheless, we are certain the mosquitoes received at least 4 logs PFU/ml of virus as determined by plaque assays conducted before and after offering mosquito an infectious blood meal. iii) *O. detritus* were reared from their habitat water while *C. quinquefasciatus* were reared in de-chlorinated water and fed on yeast. For this reason, *C. quinquefasciatus* used in this study was not the best control since larval rearing environment has significant influence on vector competence and other adult mosquito traits. However, the main purpose of the *C. quinquefasciatus* was to act as a positive control. The purpose of having *C. quinquefasciatus* was to have a way of validating that the infections were occurring successfully. Furthermore, different mosquito species may have different rearing requirements or vary in several other aspects of physiology; therefore, unless you have two strains of the same mosquito species with different vector competencies no control or comparison is ever ideal. IV) We did not record other physiological parameters such as mosquito size or daily mosquito survival of the two mosquito species. While these parameters are important they were beyond the scope of the original aim of the study, which was to assess competence of *O. detritus*. V) Finally, we used only one mosquito species in this study, despite there being several potential arboviral vectors in GB. Nevertheless, this study is one of the early contributions to the knowledgebase of vector competence of native British mosquitoes.

The vector competence studies reported here can be applied to other potential vectors. In particular, we have demonstrated that GB mosquito vector competence studies can be successfully undertaken with field-obtained specimens. Future studies will determine the vector competence of this mosquito at lower temperatures and evaluate the possibility of vertical transmission since *O. detritus* mosquitoes are available all year round and hibernate as eggs and larvae. The data provided here will prove useful for the development of GB-specific models of the risk of mosquito-borne arbovirus outbreaks in GB.

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**Figures and Tables**

**Figure 1.** Graph showing the virus eclipse phase for JEV in both *O. detritus* and *C. quinquefasciatus* at 23 °C and 28 °C. This is represented by the steady decline in number of mosquitoes with virus positive bodies from day 0 then a steady increase from Day 7 onwards.

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| Table 1. Rate of engorgement and mortality of *O. detritus* and *C. quinquefasciatus* at 23 °C and 28 °C incubation temperatures. |
| Mosquito species | **Temperature** | **Initial no. of** **mosquitoes** | **Bloodfed/****Initial (%)** | **Sampled/****Bloodfed (%)** | **Dead/****Bloodfed (%)** |
| *O. detritus* | 23 °C | 430 | 198 (46)  | 103 (52)  | 95 (48) |
|  | 28 °C | 443 | 199(45) | 70 (35) | 129 (65) |
| *C. quinquefasciatus* | 23 °C | 385 | 277(72) | 216( 78) | 61 (22) |
|  | 28 °C | 310 | 229 (74)  | 140 (61) | 89 (39) |

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| Table 2. Infection, dissemination and transmission rates of mosquitoes exposed to 4 logs PFU/ml of the Muar strain of JEV. |
| Mosquito species  | **Temp** | **dpi \*****(days)** | **No. tested** | **I** | **D** | **T** | **Infection rate a****(95% CI)** | **Dissemination rate b****(95% CI)** | **Transmission rate c****(95% CI)** |
| *O. d*  | 23°C | 0 | 16 | 16 | nt | nt | 100 (81-100) | nt | nt |
| 1 | 11 | 4 | nt | nt | 36 (15-65) | nt | nt |
| 3 | 9 | 3 | nt | nt | 33 (12-65) | nt | nt |
| 7 | 25 | 8 | 5 | 3 | 32 (17-51) | 20 (8-39) | 12 (4-30) |
| 14 | 32 | 25 | 23 | 1 | 78 (61-89) | 72 (54-84) | 3 (0-15) |
| 21 | 6 | 6 | 6 | 4 | 100 (60-100) | 100 (60-100) | 67 (30-90) |
| Total† | 63 | 39 | 34 | 8 | 62 (50-73) | 54 (42-66) | 13 (7-23) |
| 28°C | 0 | 12 | 6 | nt | nt | 50 (25-75) | nt | nt |
| 1 | 7 | 1 | nt | nt | 14 (3-51) | nt | nt |
| 3 | 3 | 0 | nt | nt | 0  | nt | nt |
| 7 | 15 | 9 | 9 | 4 | 60 (35-80) | 60 (35-80) | 27 (10-51) |
| 14 | 6 | 3 | 2 | 1 | 50 (18-81) | 33 (9-70) | 17 (3-56) |
| 21 | 3 | 3 | 3 | 1 | 100 (43-100) | 100 (43-100) | 33 (6-79) |
| Total† | 24 | 15 | 14 | 6 | 62 (42-79) | 58 (39-76) | 25 (12-45) |
| *C. q*  | 23°C | 0 | 17 | 17 | nt | nt | 100 (82-100) | nt | nt |
| 1 | 11 | 6 | nt | nt | 55 (28-55) | nt | nt |
| 3 | 11 | 3 | nt | nt | 27 (10-57) | nt | nt |
| 7 | 24 | 6 | 5 | 4 | 25 (12-44) | 21 (10-40) | 17 (7-36) |
| 14 | 32 | 20 | 11 | 1 | 62 (45-77) | 34 (20-51) | 3 (0-15) |
| 21 | 10 | 7 | 7 | 5 | 70 (39-89) | 70 (39-89) | 50 (24-76) |
| Total† | 66 | 33 | 23 | 10 | 50 (38-62) | 35 (25-47)  | 15 (8-26) |
| 28°C | 0 | 7 | 7 | nt | nt | 100 (65-100) | nt | nt |
| 1 | 10 | 3 | nt | nt | 30 (11-60) | nt | nt |
| 3 | 3 | 0 | nt | nt | 0  | nt | nt |
| 7 | 9 | 4 | 0 | 0 | 44 (19-73) | 0 | 0 |
| 14 | 12 | 8 | 7 | 2 | 66 (39-86) | 58 (31-80) | 17 (4-45) |
| 21 | 10 | 7 | 7 | 7 | 70 (40-89) | 70 (40-89) | 70 (40-89) |
| Total† | 31 | 19 | 14 | 9 | 61 (44-76)  | 45 (29-62)  | 29 (16-47)  |
| *O. d= O. detritus ; C. q= C. quinquefasciatus*; dpi=days post infection; I=number infected; D= number disseminated; T= number transmitting; nt = not tested; CI=confidence interval\* Days 0, 1, and 3 post infection represents input virus and is not true infection; † Totals include days 7 – 21 dpi onlya Percentage of mosquitoes containing virus in their bodies out of no. tested (95% confidence interval).b Percentage of mosquitoes containing virus in their legs out of no. tested (95% confidence interval).c Percentage of mosquitoes containing virus in their saliva out of no. tested (95% confidence interval). |

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| Table 3. Dissemination and transmission transition efficiency of mosquitoes exposed to 4 logs PFU/ml of Muar strain of JEV. |
| Mosquito Species  | **Temp** | **dpi (days)** | **Dissemination, % of no. infected a****(95% CI)** | **Transmission, % of no. disseminated b****(95% CI)** | **Transmission, % of no. infected c****(95% CI)** |
| *O. d*  | 23°C | 7 | 62 (30-86) | 60 (23-88) | 37 (14-69) |
| 14 | 92 (75-98) | 4 (0-21) | 4 (0-19) |
| 21 | 100 (60-100) | 67 (30-90) | 67 (30-90) |
| 28°C | 7 | 100 (70-100) | 44 (19-73) | 44 (19-73) |
| 14 | 66 (20-94) | 50 (9-90) | 33 (6-79) |
| 21 | 100 (43-100) | 33 (6-79) | 33 (6-79) |
| *C. q*  | 23°C | 7 | 83 (44-97) | 80 (38-96) | 67 (30-90) |
| 14 | 55 (34-74) | 9 (2-38) | 5 (1-24) |
| 21 | 100 (65-100) | 71 (36-92) | 71 (36-92) |
| 28°C | 7 | 0  | 0  | 0  |
| 14 | 87 (53-98) | 28 (8-64) | 25 (7-59) |
| 21 | 100 (65-100) | 100 (65-100) | 100 (65-100) |
| *O. d* = *O. detritus* ; *C. q* = *C. quinquefasciatus*; dpi= Days post infection; CI=confidence intervala Percentage of mosquitoes containing virus in their legs out of no. infectedb Percentage of mosquitoes containing virus in their saliva out of no. disseminatedc Percentage of mosquitoes containing virus in their saliva out of no. infected |

**References**

Aitken, T. (1977) An in vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. . *Mosq News,***37**, 130-133.

Boorman, J. (1987) Induction of salivation in biting midges and mosquitoes, and demonstration of virus in the saliva of infected insects. *Med Vet Entomol,***1**, 211-214.

Buckley, A., Dawson, A. & Gould, E. A. (2006) Detection of seroconversion to West Nile virus, Usutu virus and Sindbis virus in UK sentinel chickens. *Virol J,***3**, 71.

Buckley, A., Dawson, A., Moss, S. R., Hinsley, S. A., Bellamy, P. E. & Gould, E. A. (2003) Serological evidence of West Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. *J Gen Virol,***84**, 2807-2817.

Campbell, G. L., Hills, S. L., Fischer, M., Jacobson, J. A., Hoke, C. H., Hombach, J. M.*, et al.* (2011) Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ,***89**, 766-774, 774A-774E.

Chan, Y. C. & Loh, T. F. (1966) Isolation of Japanese encephalitis virus from the blood of a child in Singapore. *Am J Trop Med Hyg,***15**, 567-572.

Changbunjong, T., Weluwanarak, T., Taowan, N., Suksai, P., Chamsai, T., Sedwisai, P.*, et al.* (2013) Seasonal abundance and potential of Japanese encephalitis virus infection in mosquitoes at the nesting colony of ardeid birds, Thailand. *Asian Pac J Trop Biomed,***3**, 207-210.

Clarkson, M., J & Setzkorn, C. (2011) The domestic mosquitoes of the neston area of Chesire, UK. *Journal of the European Mosquito Control Association (European Mosquito bulletin),***29**, 122-128.

Colton, L., Biggerstaff, B. J., Johnson, A. & Nasci, R. S. (2005) Quantification of West Nile virus in vector mosquito saliva. *Journal of the American Mosquito Control Association,***21**, 49-53.

Cranston, P. S., Ramsdale, C. D., Snow, K. R. & White, G. B. (1987) *Adults, larvae and pupae of British mosquitoes (Culicidae) - a key* Freshwater Biological Association.

Davis, N. C. (1932) The effect of various temperatures in modifying the extrinsic incubation period of the yellow fever virus in *Aedes aegypti*. *American Journal of Hygiene,***16**, 163-176.

Dubrulle, M., Mousson, L., Moutailler, S., Vazeille, M. & Failloux, A. B. (2009) Chikungunya virus and Aedes mosquitoes: saliva is infectious as soon as two days after oral infection. *PLoS One,***4**, e5895.

Golding, N., Nunn, M. A., Medlock, J. M., Purse, B. V., Vaux, A. G. & Schafer, S. M. (2012) West Nile virus vector Culex modestus established in southern England. *Parasit Vectors,***5**, 32.

Halstead, S. B. & Tsai, T. F. (2004) Japanese Encephalitis Vaccines. In *Vaccines* (ed. by S. A. Plotkin & W. A. Orenstein), pp. 919-958. Saunders, Philidelphia.

Higgs, S. & Beaty, B. J. (2005) Natural cycles of vector-borne pathogens. In *Biology of Disease Vectors* (ed. by W. C. Marquardt), pp. 167-184. Elsevier, Burlington, MA.

Impoinvil, D. E., Baylis, M. & Solomon, T. (2013) Japanese encephalitis: on the one health agenda. *Current topics in microbiology and immunology,***365**, 205-247.

Johnson, N., Voller, K., Phipps, L. P., Mansfield, K. & Fooks, A. R. (2012) Rapid molecular detection methods for arboviruses of livestock of importance to northern Europe. *J Biomed Biotechnol,***2012**, 719402.

Kay, B. H., Fanning, I. D. & Mottram, P. (1989) The vector competence of Culex annulirostris, Aedes sagax and Aedes alboannulatus for Murray Valley encephalitis virus at different temperatures. *Med Vet Entomol,***3**, 107-112.

Kilpatrick, A. M. & Randolph, S. E. (2012) Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. *Lancet,***380**, 1946-1955.

Kramer, L. D. & Ebel, G. D. (2003) Dynamics of flavivirus infection in mosquitoes. *Adv Virus Res,***60**, 187-232.

Li, W. J., Wang, J. L., Li, M. H., Fu, S. H., Wang, H. Y., Wang, Z. Y.*, et al.* (2010) Mosquitoes and mosquito-borne arboviruses in the Qinghai-Tibet Plateau--focused on the Qinghai area, China. *Am J Trop Med Hyg,***82**, 705-711.

Liu, S., Zhang, Q., Zhou, J., Yu, S., Zheng, X. & Chen, Q. (2012) [Susceptibility of Aedes albopictus and Culex pipiens quinquefasciatus to infection with bat Japanese encephalitis virus isolates]. *Nan Fang Yi Ke Da Xue Xue Bao,***32**, 515-518.

Marshall, J. F. (1945) Records of Culex (Barraudius) modestus Ficalbi (Diptera,Culicidæ) obtained in the South of England. *Nature,***156**, 172-173.

Medlock, J. M., Hansford, K. M., Anderson, M., Mayho , R. & Snow, K. R. (2012) Mosquito nuisance and control in the UK - A questionnaire-based survey of local authorities. *Journal of the European Mosquito Control Association (European Mosquito bulletin),***30**, 15-29.

Medlock, J. M., Snow, K. R. & Leach, S. (2005) Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors. *Med Vet Entomol,***19**, 2-21.

Medlock, J. M. & Vaux, A. G. (2012) Distribution of West Nile virus vector, Culex modestus, in England. *Vet Rec,***171**, 278.

Medlock, J. M. & Vaux, A. G. C. (2009) Aedes (Aedes) geminus Peus (Diptera: Culicidae) an addition to the British mosquito fauna. *Dipterists Digest,***16**, 147-150.

Mohammed, M. A., Galbraith, S. E., Radford, A. D., Dove, W., Takasaki, T., Kurane, I.*, et al.* (2011) Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus reveal it is the missing fifth genotype. *Infect Genet Evol,***11**, 855-862.

Mourya, D. T., Gokhale, M. D., Pidiyar, V., Barde, P. V., Patole, M., Mishra, A. C.*, et al.* (2002) Study of the effect of the midgut bacterial flora of Culex quinquefasciatus on the susceptibility of mosquitoes to Japanese encephalitis virus. *Acta Virol,***46**, 257-260.

Newcombe, R. G. (1998) Two-sided confidence intervals for the single proportion: comparison of seven methods. *Stat Med,***17**, 857-872.

Nitatpattana, N., Apiwathnasorn, C., Barbazan, P., Leemingsawat, S., Yoksan, S. & Gonzalez, J. P. (2005) First isolation of Japanese encephalitis from Culex quinquefasciatus in Thailand. *Southeast Asian J Trop Med Public Health,***36**, 875-878.

Osorio, H. C., Ze-Ze, L. & Alves, M. J. (2012) Host-feeding patterns of Culex pipiens and other potential mosquito vectors (Diptera: Culicidae) of West Nile virus (Flaviviridae) collected in Portugal. *J Med Entomol,***49**, 717-721.

Phipps, L. P., Duff, J. P., Holmes, J. P., Gough, R. E., McCracken, F., McElhinney, L. M.*, et al.* (2008) Surveillance for West Nile virus in British birds (2001 to 2006). *Vet Rec,***162**, 413-415.

Platonov, A. E., Rossi, G., Karan, L. S., Mironov, K. O., Busani, L. & Rezza, G. (2012) Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds collected in Italy. *Eurosurveillance,***17**.

Ravanini, P., Huhtamo, E., Ilaria, V., Crobu, M. G., Nicosia, A. M., Servino, L.*, et al.* (2012) Japanese encephalitis virus RNA detected in Culex pipiens mosquitoes in Italy. *Euro Surveill,***17**.

Rees, A. T. & Snow, K. R. (1996) The distribution of *Aedes*: subgenus *Ochlerotatus* in Britain. . *Dipterists Digest (Second Series),***3**, 5-23.

Richards, S. L., Pesko, K., Alto, B. W. & Mores, C. N. (2007) Reduced infection in mosquitoes exposed to blood meals containing previously frozen flaviviruses. *Virus research,***129**, 224-227.

Scherer, W. F., Kitaoka, M., Okuno, T. & Ogata, T. (1959) Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. *Am J Trop Med Hyg,***8**, 707-715.

Service, M. W. (1971) Feeding behaviour and host preferences of British mosquitoes. *Bulletin of Entomological Research,***60**, 653-661.

Snow, K. R. (1990) *Mosquitoes*. Richmond Publishing Company, London.

Snow, K. R., Rees, A. T. & Bulbeck, S. J. (1998) *A Provisional Atlas of the Mosquitoes of Britain*. University of East London, London.

Takahashi, M. (1976) The effects of environmental and physiological conditions of Culex tritaeniorhynchus on the pattern of transmission of Japanese encephalitis virus. *J Med Entomol,***13**, 275-284.

Turell, M. J., Gargan, T. P., 2nd & Bailey, C. L. (1984) Replication and dissemination of Rift Valley fever virus in Culex pipiens. *Am J Trop Med Hyg,***33**, 176-181.

Turell, M. J., Mores, C. N., Dohm, D. J., Lee, W. J., Kim, H. C. & Klein, T. A. (2006) Laboratory transmission of Japanese encephalitis, West Nile, and Getah viruses by mosquitoes (Diptera: Culicidae) collected near Camp Greaves, Gyeonggi Province, Republic of Korea 2003. *J Med Entomol,***43**, 1076-1081.

Unni, S. K., Ruzek, D., Chhatbar, C., Mishra, R., Johri, M. K. & Singh, S. K. (2011) Japanese encephalitis virus: from genome to infectome. *Microbes Infect,***13**, 312-321.

van den Hurk, A. F., Nisbet, D. J., Hall, R. A., Kay, B. H., MacKenzie, J. S. & Ritchie, S. A. (2003) Vector competence of Australian mosquitoes (Diptera: Culicidae) for Japanese encephalitis virus. *J Med Entomol,***40**, 82-90.

van den Hurk, A. F., Ritchie, S. A. & Mackenzie, J. S. (2009) Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol,***54**, 17-35.

Weng, M. H., Lien, J. C., Wang, Y. M., Lin, C. C., Lin, H. C. & Chin, C. (1999) Isolation of Japanese encephalitis virus from mosquitoes collected in Northern Taiwan between 1995 and 1996. *J Microbiol Immunol Infect,***32**, 9-13.