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Rift Valley fever virus and European mosquitoes: vector competence of *Culex pipiens* and *Stegomyia albopicta* (=*Aedes albopictus*)

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Abstract. Rift Valley fever (RVF) is a mosquito-borne disease caused by the Rift Valley fever virus (RVFV). Rift Valley fever affects a large number of species, including human, and has severe impact on public health and the economy, especially in African countries. The present study examined the vector competence of three different European mosquito species, *Culex pipiens* (Linnaeus, 1758) form *molestus* (Diptera: Culicidae), *Culex pipiens* hybrid form and *Stegomyia albopicta* (=*Aedes albopictus*) (Skuse, 1894) (Diptera: Culicidae). Mosquitoes were artificially fed with blood containing RVFV. Infection, disseminated infection and transmission efficiency were evaluated. This is the first study to assess the transmission efficiency of European mosquito species using a virulent RVFV strain. The virus disseminated in *Cx. pipiens* hybrid form and in *S. albopicta*. Moreover, infectious viral particles were isolated from saliva of both species, showing their RVFV transmission capacity. The presence of competent *Cx. pipiens* and *S. albopicta* in Spain indicates that an autochthonous outbreak of RVF may occur if the virus is introduced. These findings provide information that will help health authorities to set up efficient entomological surveillance and RVFV vector control programmes.

Key words. *Culex pipiens*, *Stegomyia albopicta* (=*Aedes albopictus*), FTA™ Cards, Rift Valley fever virus, saliva, transmission, vector competence.

Introduction

Rift Valley fever (RVF) is an arthropod-borne zoonotic disease caused by Rift Valley fever virus (RVFV), an arbovirus of the *Phlebovirus* genus, belonging to the Bunyaviridae family. Rift Valley fever is a zoonotic disease transmitted by infected mosquitoes to a wide range of hosts, including both domestic (especially sheep and goat) and wild (African buffalo, waterbuck, camel, rat) animals (Olive *et al.*, 2012). Rift Valley fever virus has been isolated in more than 50 mosquito species from seven different genera, although the majority belong to the *Culex* and *Stegomyia* (*Aedes*) genera (Linthicum *et al.*, 2016). The virus was first described in 1931 in the Rift Valley province of Kenya (Daubney *et al.*, 1931). Since then, RVFV has caused significant human and animal outbreaks in several African countries, including South Africa (1950–1951, 2008–2011), Egypt (1977, 1997, 2003), Mayotte (2007–2008), Madagascar (2008), Kenya (1997–1998, 2006–2007), Tanzania (2007), Somalia (2007) and Mauritania (2010–2011, 2013–2014) (Gerdes, 2004; Nanyingi *et al.*, 2015; Linthicum *et al.*, 2016; Métras *et al.*,

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2016). In 2000, RVFV was reported for the first time outside the African continent, in Saudi Arabia and Yemen (Ahmad, 2000). The impact of RVFV on public health and the economy can be very high, as was reported after the epidemic outbreak in Saudi Arabia in 2000. During this outbreak, 883 people were infected, resulting in 124 human deaths, and 40 000 animals died or were aborted (Al-Afaleq & Hussein, 2011). Another important outbreak occurred in Egypt during 1977, during which 200 000 human clinical cases, 600 deaths and economic losses of more than US$115 m were reported (Meegan *et al.*, 1980).

The presence of RVFV outside the African continent and especially in countries bordering the Mediterranean Sea, such as Egypt, highlights the possibility that RVF may be introduced into Europe. The risk for the introduction of RVFV into Europe has been reviewed (Chevalier *et al.*, 2010; Rolin *et al.*, 2013; Mansfield *et al.*, 2015). These authors classified the risk for the introduction of RVFV into countries within the European Union as low, mainly because of restrictions imposed by the EU on the import of livestock, differences in climate and seasonal variations in vector and host densities in comparison with those present in Africa. Nonetheless, the illegal importation of infected livestock, especially between Africa and southern Europe and between the Middle East and central Europe, has been indicated as the most likely source of virus introduction into Europe (Chevalier *et al.*, 2010). Climate is a key factor in estimating the risk for RVF outbreaks (Gerdes, 2004). The unusual strength of *El Niño* and the consequent rainfall anomalies reported have enhanced the risk for further RVFV outbreaks in many African countries [Food & Agriculture Organization, Office International des Épizooties & World Health Organization (FAO, OIE & WHO), 2015; U.S. Department of Agriculture (USDA), 2015] as a result of increases in vector density, an important parameter used to estimate vectorial capacity (Garrett-Jones, 1964; Smith *et al.*, 2012). The climatic effects of *El Niño* are also expected to affect several European countries with rainfall anomalies (USDA, 2015). Furthermore, several unpredictable factors, such as bioterrorism and the intentional introduction of the virus may increase the risk for RVFV introduction, but entomological research and knowledge of vector ecology may improve estimations of risk for RVF (Rolin *et al.*, 2013). Perceptions of the risk for the introduction of RVFV into Europe are further enhanced by evidence from countries in northwest Africa (Mauritania and Senegal) in which outbreaks have been reported (Nanyingi *et al.*, 2015), serological evidence of RVFV antibodies in camels in Morocco (El-Harrak *et al.*, 2011) and the presence of stable competent vector populations in Algeria, Morocco and Tunisia (Moutailler *et al.*, 2008; Amraoui *et al.*, 2012).

Mosquitoes belonging to the *Culex pipiens* complex are known to be efficient vectors of RVFV (Turell *et al.*, 1996) and have been proposed as the principal vectors during the 1977 outbreak in Egypt (Meegan *et al.*, 1980). Mosquito species of the *Culex* genus (*Culex theileri* Theobald, *Culex perexiguus* Theobald and *Culex antennatus*) have also been considered as potential vectors as a result of their bio-ecology in terms of abundance, biting activity, feeding habits and longevity [European Food Safety Authority (EFSA), 2013]. Despite the scant data on the possible role of *Stegomyia albopicta* (=*Aedes albopictus*) as a vector of RVFV, studies on its host-feeding patterns in rural areas (Valerio *et al.*, 2009; Faraji *et al.*, 2014) would suggest that this species could contribute to RVFV transmission.

Other characteristics of *Stegomyia* mosquitoes are particularly relevant and may suggest an active role of *S. albopicta* in RVFV transmission: *Stegomyia* mosquitoes are able to transmit RVFV transovarially (Linthicum *et al.*, 1985); moreover, the eggs of *Stegomyia* spp. may enter diapause and survive at temperatures between 0 °C and −15 °C (Thomas *et al.*, 2012). For these reasons, *S. albopicta* may be important not only for its ability to horizontally transmit the virus, but also for its capacity to maintain viable virus during the coldest winter months. *Culex pipiens* and *S. albopicta* are expected to be the main vector species because of their massive presence within the countries of the Mediterranean basin.

To date, only one study of vector competence has been performed in European mosquitoes (Moutailler *et al.*, 2008). The authors of this study tested the capacity of two different strains of RVFV (the virulent strain ZH548 and the avirulent strain Clone 13) to produce disseminated infections in several mosquito species from the Camargue region of France [*Ochlerotatus caspius* Pallas (=*Aedes caspius*) (Diptera: Culicidae), *Ochlerotatus detritus* (=*Aedes detritus*) Haliday and *Cx. pipiens*]. In other regions of the world, RVFV vector competence studies have been more exhaustive. In Africa, nine species have been tested as vectors of RVFV, including *Stegomyia aegypti* Linnaeus (=*Aedes aegypti* Linnaeus), *Stegomyia calceata* Edwards (=*Aedes calceata* Edwards), *Aedes circumluteolus* Theobald, *Aedes mcintoshi* Huang, *Aedes palpalis* Taylor, *Cx. antennatus* Becker, *Cx. pipiens*, *Culex quinquefasciatus* Say and *Culex zombaensis* Theobald (Turell *et al.*, 1996, 2007, 2008a; Moutailler *et al.*, 2008; Amraoui *et al.*, 2012). Further, nine species have been tested in Canada (Iranpour *et al.*, 2011), four species in Australia (Turell & Kay, 1998) and 21 species in the U.S.A. (Turell *et al.*, 1988, 2008b, 2010, 2013a, 2013b, 2015).

Given the presence of potential vectors and favourable environmental conditions in some areas (Sanchez-Vizcaino *et al.*, 2013), the possibility of an RVF outbreak event in Spain cannot be excluded. In this context, the vector competence (infection, dissemination and transmission) of two different strains of *Cx. pipiens* and a strain of *S. albopicta* were investigated using a virulent strain of RVFV. To the present authors' knowledge, this is the first study to test transmission efficiency in European species using a virulent RVFV strain. Two different approaches involving FTA™ cards and capillary techniques were compared to test viral transmission. Furthermore, experimental mosquito infections were achieved using cycling conditions that simulated environmental conditions. This should allow for a more realistic estimation of vector competence in mosquitoes present in Europe.

Materials and methods

Mosquito strains

Two different strains of *Cx. pipiens* were used. These included a *Cx. pipiens* form *molestus* from Empuriabrava (2011) and a hybrid between the *pipiens* form and *molestus* form, from Gavà (2012). Molecular characterization of the *Cx. pipiens* forms was performed as previously described (Bahnck & Fonseca, 2006). Empuriabrava is located near the Aiguamolls de l'Empordà, a wetland area in the north of Catalonia. Gavà is a coastal tourist town within the metropolitan area of Barcelona. The strain of *S. albopicta* was sourced in 2005 from Sant Cugat de Vallés, a town within the metropolitan area of Barcelona and the site of the first finding of an identified Asian tiger mosquito in Spain in 2004 (Aranda *et al.*, 2006). Mosquitoes were reared under a 14 : 10 h (light:dark) photoperiod with two crepuscular cycles of 30 min inserted to simulate dawn and dusk; mean temperature was 26 °C during the day and 22 °C during the night. Relative humidity (RH) was maintained at a constant 80%. These environmental conditions corresponded to the mean temperature and photoperiod at the latitude of the study area (41°24′ N, 2°10′ E) during summer (July and August), when the density and activity of mosquitoes are expected to be at their highest.

The mosquito strains were tested for the presence of viruses from the genera *Flavivirus* (family Flaviviridae), *Alphavirus* (family Togaviridae) and *Phlebovirus* (family Bunyaviridae) by reverse transcription nested polymerase chain reaction (RT-nPCR) (Sánchez-Seco *et al.*, 2001, 2003, 2005) to confirm the absence of other viral infections. The strains were also tested for the presence of *Wolbachia* spp. by PCR analysis of a fragment of *wsp* gene as previously described (Braig *et al.*, 1998). All strains were positive for *Wolbachia* spp. but negative for *Flavivirus*, *Alphavirus* and *Phlebovirus* (data not shown).

Virus strains

The virulent strain RVF 56/74, originally isolated from cattle in 1977 (Barnard & Botha, 1977), was used in the present study. RVF 56/74 was propagated in baby hamster kidney fibroblast 21 (BHK-21) cells (Busquets *et al.*, 2010) and titrated to obtain the 50% tissue culture infective dose per mL ($TCID_{50}$/mL) in African green monkey kidney (Vero) cells.

Design of vector competence assays

Infection rate (IR), disseminated infection rate (DIR) and transmission efficiency (TE) were evaluated. The IR is defined as the proportion of mosquitoes in which the body (abdomen, thorax and head) is infected among all tested mosquitoes. In these mosquitoes, the virus was able to overcome the midgut infection barrier (MIB). The DIR is defined as the proportion of mosquitoes in which the legs and wings are infected among all mosquitoes in which the body is infected. In these mosquitoes, the virus was able to overcome midgut infection and escape barriers. The TE is defined as the proportion of mosquitoes with infectious saliva among the total number of mosquitoes tested (Jupille *et al.*, 2016). In these mosquitoes, the virus was able to overcome the salivary glands infection and escape barriers.

Three assays were performed. In the first assay, the two forms of *Cx. pipiens* were tested using two different viral doses: 5.7 $\log_{10}TCID_{50}$/mL and 7.0 $\log_{10}TCID_{50}$/mL. This first trial was designed to elucidate the IR and DIR of the two forms of *Cx. pipiens*, mimicking low and medium–high viraemia. The presence of viral RNA in saliva was evaluated using FTA™ Cards (GE Healthcare, Little Chalfont, U.K.). In the second and third assays, two different approaches were used to test saliva samples, using, respectively, FTA™ Cards and a capillary for the direct extraction of saliva from the mosquito. In the second assay, the *Cx. pipiens* hybrid strain was tested using a viral dose of 7.5 $\log_{10}TCID_{50}$/mL. In the third assay, the *S. albopicta* strain was tested using a viral dose of 6.2 $\log_{10}TCID_{50}$/mL.

In all assays, female mosquitoes aged 7–9 days that had never blood fed were used. Mosquitoes were housed in 0.5-L plastic cages with mesh screening and fed on a 10% sucrose solution *ad libitum*. The sucrose solution was removed 30 h before the mosquitoes were given infectious bloodmeals. The mosquitoes were fed using a Hemotek feeding system (Discovery Workshop, Accrington, U.K.) at 38 ± 0.5 °C with a specific pathogen-free chicken skin as a membrane (Valo BioMedia GmbH, Osterholz-Scharmbeck, Germany). The mosquitoes were fed with heparinized bovine blood (Universitat Autònoma de Barcelona, Barcelona, Spain) doped with RVFV (maximum virus:blood ratio: 1 : 3) and adenosine 5′-triphosphate (ATP) (5×10^{-3} M) (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). After feeding on the infected blood, the mosquitoes were anaesthetized with carbon dioxide (CO_2) and fully engorged females were selected. The infected blood was titrated in Vero cells; some specimens from each group were killed and analysed to provide inoculum control. The rest of the mosquitoes were individually transferred to cardboard cages (Watkins & Doncaster, Leominster, U.K.) sealed with mesh screening on top and stored inside a climatic cabinet under the environmental conditions described above. Sucrose solution was administered on soaked cotton pledgets placed on the mesh screen and changed every day. All assays were performed in Biosafety Level 3 facilities at the Centre de Recerca en Sanitat Animal (CReSA).

Sample collection

In all assays, FTA™ Cards were used to take saliva samples at different time-points, including at 14 days post-infection (d.p.i.) in the first assay, and at 5 d.p.i. and 14 d.p.i. in the second and third experiments, respectively. FTA™ Cards were soaked in Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zealand) and a blue alimentary colorant. The FTA™ Cards were left for 24 h on the top of the mesh screen of all individual cardboard cages to allow the mosquitoes to feed from the cards. Subsequently, the FTA™ Cards were collected, re-suspended in 0.3 mL of phosphate-buffered saline (PBS) and stored at −80 °C until tested.

At 14 d.p.i., each mosquito was anaesthetized with CO_2 and dissected. The legs and wings were detached from the body and both parts were separately homogenized in 0.5 mL of Dulbecco's modified Eagle's medium (DMEM) (Lonza Group AG, Basel, Switzerland). The samples were homogenized at 30 Hz for 1 min using TissueLyser II (Qiagen GmbH, Hilden, Germany) and stored at −80 °C until tested for RVFV.

In the second and third trials, saliva was extracted from each mosquito at 14 d.p.i. using a capillary technique, as previously

Table 1. Feeding and mortality rates.

	First assay				Second assay		Third assay	
Viral dose	5.7 $\log_{10}$TCID$_{50}$/mL		7.0 $\log_{10}$TCID$_{50}$/mL		7.5 $\log_{10}$TCID$_{50}$/mL		6.2 $\log_{10}$TCID$_{50}$/mL	
Species	FEF	Mortality	FEF	Mortality	FEF	Mortality	FEF	Mortality
Culex pipiens form *molestus*	5.2% $n = 211*$	12.5% $n = 8$	2.4% $n = 329*$	0% $n = 5$	NA	NA	NA	NA
Culex pipiens hybrid	22.2% $n = 332*$	3.0% $n = 66$	33.3% $n = 294*$	2.5% $n = 40$	21.4% $n = 312*$	4.6% $n = 65$	NA	NA
Stegomyia albopicta	NA	NA	NA	NA	NA	NA	24.5% $n = 200*$	7.3% $n = 41$

*Some females were selected immediately after feeding and analysed as inoculum control.
FEF, fully engorged female; NA, not applicable; TCID$_{50}$, 50% tissue culture infective dose.

described (Dubrulle *et al.*, 2009). Briefly, after the dissection of the legs and wings, the proboscis was inserted into a P20 pipette tip filled with 7 µL of a 1 : 1 solution of fetal bovine serum (FBS) and 50% sucrose solution. To stimulate salivation, 1 µL of 1% pilocarpine (Sigma-Aldrich Corp.), prepared in PBS at 0.1% Tween 80, was applied to the thorax of each mosquito. After 60 min, the solution containing the saliva was expelled into 1.5-mL tubes containing 193 µL of DMEM; 150 µL were used for viral RNA extraction and the remaining samples were used for RVFV isolation in a monolayer of Vero cells. Cells were incubated for 7 days (37 °C, 5% CO$_2$) and cytophatic effects were evaluated.

Virus detection

Viral RNA was extracted from samples (bodies, legs and wings, FTA™ Cards and saliva) using NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations. The RT-PCR was performed as previously described with minor modifications (Drosten *et al.*, 2002). Reverse transcription quantitative PCR (RT-qPCR) was carried out using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, Inc., Foster City, CA, U.S.A.) without adding supplementary MgSO$_4$. The samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.) programmed as follows: 48 °C for 10 min; 95 °C for 10 min, and 45 cycles at 95 °C for 15 s and at 57 °C for 35 s. The limit of detection was estimated at 0.09 TCID$_{50}$ per reaction.

Results

Mosquito feeding and mortality

The two forms of *Cx. pipiens* behaved differently during artificial feeding; data are shown in Table 1. The mean ± standard deviation (SD) feeding rate was higher in *Cx. pipiens* hybrids (25.6 ± 6.62%) than in the *Cx. pipiens* form *molestus* strain (3.8 ± 1.96%). Mean ± SD mortality rates at 14 d.p.i. were 3.3 ± 1.10% and 6.2 ± 8.83% in *Cx. pipiens* hybrids and *Cx. pipiens* form *molestus*, respectively. In *S. albopicta*, the feeding rate was 24.5% and the mortality rate at 14 d.p.i. was 7.3%.

Mosquito infection and dissemination

Infected mosquito bodies were detected for both *Cx. pipiens* forms tested using the lowest viral dose (5.7 $\log_{10}$ TCID$_{50}$/mL). However, disseminated infection was not detected in any mosquito. By contrast, a viral dose of 7.0 $\log_{10}$ TCID$_{50}$/mL was able to induce both infection and disseminated infection in the *Cx. pipiens* hybrid form. At the same viral dose, C*x. pipiens* form *molestus* presented infection, but not dissemination. Both infection and disseminated infection were detected in *S. albopicta*. The IRs and DIRs are summarized in Table 2. The virus was able to cross the midgut barriers in mosquitoes of the *Cx. pipiens* hybrid form and *S. albopicta* showing disseminated infection (positive legs and wings).

Transmission of RVFV

Rift Valley fever virus was detected in saliva of the *Cx. pipiens* hybrid form and *S. albopicta* using both FTA™ Cards and the capillary technique. A positive saliva sample indicates that the virus was able to cross salivary gland barriers. All positive saliva samples (FTA™ Cards and collected saliva) are reported in Table 3 and are related to the corresponding samples of legs and wings and to the results of isolation in Vero cells (presence/absence of cytophatic effects).

In the first assay, no FTA™ Cards tested positive, regardless of the titre used or the mosquito strain tested.

In the second assay, two FTA™ Cards tested positive. These FTA™ Cards referred to two different specimens of *Cx. pipiens* hybrid form (M-46 and M-49) and were sampled at different time-points (5 and 14 d.p.i.). Neither mosquito presented disseminated infection. Five saliva samples obtained using the capillary technique in *Cx. pipiens* hybrids tested positive by RT-qPCR. Three of these produced cytophatic effects when inoculated in Vero cells. Two specimens (M-14 and M-59) with infectious viral particles in saliva presented disseminated infection. Conversely, specimen M-5 did not present disseminated

Table 2. Infection rate (IR), disseminated infection rate (DIR) and estimated presence of midgut infection and escape barriers.

	First assay				Second assay		Third assay	
	5.7 $\log_{10}$TCID$_{50}$/mL		7.0 $\log_{10}$TCID$_{50}$/mL		7.5 $\log_{10}$TCID$_{50}$/mL		6.2 $\log_{10}$TCID$_{50}$/mL	
Species	IR (MIB)	DIR (MEB)	IR (MIB)	DIR (MEB)	IR (MIB)	DIR (MEB)	IR (MIB)	DIR (MEB)
Culex pipiens form *molestus*	14.2% $n=7$ ++++	0% $n=1$ ++++*	20.0% $n=5$ +++*	0% $n=3$	NA	NA	NA	NA
Culex pipiens hybrid	12.6% $n=63$ ++++	0% $n=8$ ++++	7.0% $n=39$ ++++	66.6% $n=3$ +*	29.0% $n=62$ +++	33.3% $n=18$ +++	NA	NA
Stegomyia albopicta	NA	NA	NA	NA	NA	NA	10.5% $n=38$ ++++	25.0% $n=4$ +++*

*Questionable, based on the small sample size.

MEB, midgut escape barrier; MIB, midgut infection barrier; NA, not applicable; TCID$_{50}$, 50% tissue culture infective dose.

Rating: +, minor, virus crosses this barrier in 60–80% of mosquitoes; ++, moderate, virus crosses this barrier in 40–60% of mosquitoes; +++, severe, virus crosses this barrier in 20–40% of mosquitoes, ++++, very severe, virus crosses this barrier in < 20% of mosquitoes.

Table 3. Presence of virus in different samples of mosquitoes with positive saliva.

Mosquito	Legs and wings	FTA 5 d.p.i.	FTA 14 d.p.i.	Saliva 14 d.p.i.	Saliva 14 d.p.i./cytophatic effect
M-5	−	−	−	+ (36.64)	+
M-14	+ (23.30)	−	−	+ (30.40)	+
M-35	+ (26.39)	−	−	+ (35.68)	−
M-46	−	+ (39.88)	−	+ (39.47)	−
M-49	−	−	+ (30.73)	−	−
M-59	+ (23.20)	−	−	+ (29.49)	+
V-3	+ (26.95)	+ (36.53)	−	+ (30.32)	+

d.p.i., days post-infection; M-n, *Culex pipiens* hybrid, second assay; V-n, *Stegomyia albopicta*, third assay; −, negative; +, positive.

Ct values of positive samples analysed by reverse transcription quantitative polymerase chain reaction are reported in brackets.

infection. The FTA™ Cards for these three specimens were negative.

In summary, two patterns of transmission were evidenced in *Cx. pipiens*, in, respectively, females that were able to transmit the virus without presenting disseminated infection, and females that transmitted the virus and presented disseminated infection.

In the third assay, one FTA™ Card referred to an *S. albopicta* specimen with disseminated infection (V-3) tested positive at 5 d.p.i., although the FTA™ Card tested negative at 14 d.p.i. However, the saliva sample extracted at 14 d.p.i. from the same mosquito tested positive by RT-PCR and was able to induce a cytophatic effect when inoculated in Vero cells.

Transmission efficiency was estimated at 4.8% (three mosquitoes with infectious saliva out of 62 mosquitoes fed) in *Cx. pipiens* hybrids and 2.6% (one of 38 mosquitoes) in *S. albopicta*.

Discussion

Mosquitoes belonging to *Culex* and *Stegomyia* (= *Aedes*) spp. are considered to be the main vectors for RVFV. In the present study, for the first time, two strains of different *Cx. pipiens* forms and one strain of *S. albopicta* collected in Spain were demonstrated to be susceptible to RVFV infection. Moreover, *Cx. pipiens* hybrids and *S. albopicta* were able to transmit RVFV.

Culex pipiens form *molestus* exhibited a lower propensity to feed from the artificial feeding system used, and was not found to be a useful laboratory species for vector competence studies.

In the *Cx. pipiens* hybrid form, rates of infection and dissemination tended to increase proportionally to the viral dose used during blood feeding, as previously observed in several species (Turell *et al.*, 2008b, 2013b). This finding probably reflects the presence of dose-dependent midgut barriers which the virus must overcome in order to successfully infect and disseminate to the whole mosquito body (Franz *et al.*, 2015). A previous study, performed with the RVFV strain ZH501, suggested the presence of a midgut escape barrier (MEB) in *Cx. pipiens* form *molestus* (Turell *et al.*, 2014). This may explain the absence of disseminated infection in this species in the present study, but the low number of *Cx. pipiens* form *molestus* that fed successfully did not provide sufficient data to strongly support this hypothesis and thus further studies are required to clarify this point.

Rates of infection and dissemination in Spanish *Cx. pipiens* were lower than those in *Cx. pipiens* tested in France (Moutailler *et al.*, 2008) and Canada (Iranpour *et al.*, 2011). A comparison of the experimental procedures used in the earlier studies with those used in the present study shows some significant differences: (a) the titres used for challenges were higher [$10^{7.9}$–$10^{9.4}$ plaque-forming units (PFU)/mL]; (b) the source of feeding was a live infected hamster, and (c) the experimental procedures were conducted under a constant temperature of 28 °C in the French study and 25 °C in the Canadian study. As mentioned before, viral dose directly influences both infection and dissemination rates. The use of a living host for feeding improves the competence of the mosquito specimens tested (Turell, 1988; Lord *et al.*, 2006). In the present study, the viral doses used corresponded to the viral loads detected in blood from European lambs experimentally infected with the same virulent RVFV strain (Busquets *et al.*, 2010). It is known that a higher and constant extrinsic incubation temperature (EIT) corresponds to high rates of infection, dissemination and transmission, as has been experimentally demonstrated for different arboviruses (Richards *et al.*, 2007; Kilpatrick *et al.*, 2008; Lambrechts *et al.*, 2011). A previous study on the effect of EIT on the vector competence of *Cx. quinquefasciatus* for West Nile virus (WNV) suggested that EIT can influence both the MIB and MEB (Anderson *et al.*, 2010). A more recent study found that the cycling of environmental conditions can also affect vector competence for WNV in *Cx. pipiens* and *S. albopicta* (Brustolin *et al.*, 2016). Cycling of environmental conditions directly affects vector competence in the strains tested and hence was applied in the present study to mimic environmental conditions in the field in order to better estimate vector competence. With regard to the influence of the viral load used, the findings of the present study can be compared with those of a previous study in which two forms of *Cx. pipiens* from the U.S.A. were assayed using a similar viral load ($10^{7.5}$ PFU/mL) (Turell *et al.*, 2014). However, the authors of the U.S. study used an infected hamster as a blood source and specimens were maintained at a constant EIT of 26 °C (Turell *et al.*, 2014). As result, the infection and dissemination rates obtained were higher than those in the present study.

With regard to RVFV transmission, the present results show that the Spanish *S. albopicta* and the *Cx. pipiens* hybrid form strains could possibly sustain the RVFV transmission cycle in nature. One positive FTA™ Card at 5 d.p.i. provided evidence of early transmission capacity in the *Cx. pipiens* hybrid form, as previously observed in *Cx. pipiens* from the Maghreb region (Amraoui *et al.*, 2012). In the Maghreb populations, the presence of infectious viral particles was observed from 3 d.p.i

The rates of RVFV infection, dissemination and transmission observed in the Spanish *S. albopicta* strain are comparable with those obtained in a previous vector competence study conducted in *S. albopicta* mosquitoes from Texas (Turell *et al.*, 1988) fed with an infectious bloodmeal at a final titre of $10^{4.7}$ PFU. The finding of a positive FTA™ Card at 5 d.p.i. also showed early transmission capacity, which contrasts with that described previously for the Texas specimens, which were able to transmit RVFV only at 14 d.p.i

The FTA™ Card was originally designed as a surveillance tool for arbovirus detection in field studies and was intended to avoid the analysis of trapped vectors (Van den Hurk *et al.*, 2012). The exposure period for FTA™ Cards was 7 days in field studies. It is probable that a shorter period of exposure will limit the possibility that mosquitoes will feed on the card, which will result in a lower number of positive FTA™ Cards compared with the number of positive saliva samples obtained by capillary extraction. The presence of a blue-coloured belly indicated that the specimen had fed from an FTA™ Card soaked in honey. Negative FTA™ Cards from mosquitoes with positive saliva mainly corresponded to specimens without a blue belly, although the blue belly was not always evident to the naked eye. However, some authors have suggested that forcing salivation in a capillary for 30–45 min may produce an inaccurate overestimation of viral transmission (Smith *et al.*, 2006). The differences in the results obtained by FTA™ Cards and those obtained in saliva directly extracted with the capillary technique are likely to reflect several factors: (a) lower sensitivity of the FTA™ Card technique; (b) an insufficient period of exposure of the FTA™ Card, and (c) an overestimation of viral shed in the capillary.

Two *Cx. pipiens* hybrid mosquitoes (M-5 and M-49) with positive saliva samples, but without dissemination infection, were observed. Previous studies have described the possibility that RVFV might disseminate from the midgut via the trachea (Romoser *et al.*, 2005; Kading *et al.*, 2014). This would provide a direct pathway to the salivary gland without the need for dissemination in haemocoel and other secondary target organs. Therefore, two patterns of RVFV transmission in females are reported: transmission in females with disseminated infection, and transmission in females without disseminated infection. Further experiments with higher numbers of RVFV-infected mosquitoes are required to strengthen this model.

The three strains of mosquito used in the present study were all naturally infected by *Wolbachia* spp. This may have influenced the vector competence of infected mosquitoes, as has been shown in previous studies (Moreira *et al.*, 2009; Walker *et al.*, 2011). However, further studies regarding this issue are required to elucidate its possible role in arbovirus–vector interactions.

The risk for the introduction of RVFV into regions of Spain in which livestock densities are high and environmental conditions are favourable has been analysed in a previous study (Sanchez-Vizcaino *et al.*, 2013). Several Spanish regions, including Catalonia, were found to be suitable for an RVF outbreak. The findings related to RVFV vector competence presented in the current work would support this possibility as both Spanish *S. albopicta* and *Cx. pipiens* hybrid strains appear to be able to sustain the cycle of RVFV transmission.

In conclusion, the data presented in this work provide information that will help in the establishing of effective vector control programmes and surveillance plans to prevent and control possible RVF outbreaks. Additional studies are required to evaluate the vector competence of other European autochthonous vectors and their possible roles during an RVF outbreak.

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