Experimental bluetongue virus infection of Culicoides austropalpalis*, collected from a farm environment in Victoria, Australia*

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Summary
Following the emerging bluetongue virus transmission in European temperate regions, we question the vector competence of the abundant *Culicoides austropalpalis* Lee and Reye in South-East temperate Australia. Field collected *Culicoides* midges were membrane fed with a bluetongue virus serotype 1 (BTV-1). The average feeding rate was 50%. After 13 days, survival rate was 25% and virus RNA presence was checked by quantitative PCR targeting viral genome segment 10. Virus RNA was found in 7.4% of individually tested females with relative viral RNA load values lower than freshly fed females, indicating that viral replication was low or null. A second qPCR targeting viral genome segment 1 confirmed the presence of virus RNA in only four out of 29 previously positive specimens. After 10 days culture on *Culicoides* cells, none of these four confimed positive samples did show subsequent cytopathogenic effect on Vero cells or BTV antigen detection by ELISA. As control for this virus activity detection, 12 days after microinjection of BTV-1, *Culex annulirostris* mosquitoes showed, after culture on Kc cells, cytopathogenic effect on Vero cells, with ELISA-confirmed infection. Despite its abundance in farm environment of the temperate Australian regions, the results of this study make *C. austropalpalis* of unlikely epidemiological importance in the transmission of BTV in Australia.

Bluetongue (BT) is a debilitating arbovirus infection of sheep and sometimes cattle, transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae) with other domestic and wild ruminants serving as asymptomatic reservoirs, mostly. Multiple serotypes of bluetongue virus (BTV) are enzootic in northern and eastern Australia (Firth *et al.* 2017). However, there has been no evidence to date of naturally occurring BT in Australian livestock. In 2006, BTV serotype 8 (BTV-8) emerged unexpectedly in northern Europe. Although the source of this virus is unclear, BTV-8 adapted to local Palaearctic *Culicoides* species, successfully over-wintered, and became endemic across part of northern Europe (Carpenter *et al.* 2009). These events suggested that the balance of factors that have allowed Australia to remain free of the disease may be relatively fragile and could be disrupted by the invasion of exotic vectors or more virulent viruses from Asia, changes in the distribution of endemic viruses or vectors, and/or adaptation of viruses to vectors with a more southerly distribution. The invasion of BTV and/or its vectors into southern sheep farming zones of Australia would have significant impacts both on animal health and the export trade. There are critical gaps in our understanding of the genetics, biology,

ecology and distribution of BTV and *Culicoides* in Australia, and the potential consequences of global warming. For a better understanding of the BTV transmission potential of the *Culicoides* species present in the southern parts of Australia, we have performed BTV experimental infection of *Culicoides* caught on a farm in the southern Australian state of Victoria.

The collection and infection procedures are detailed in Venter and colleagues (Venter *et al.* 1998). Briefly, Onderstepoort UV traps were set up at a farm at Benalla, Victoria (145.9 E, 36.6 S, 170 m above sea level) for 33 trap-nights during March 2014. Three to four traps were set per night close to resting animals (sheep, horses) in paddocks. Insects were collected alive and self-sorted by exiting through meshed funnel into small cardboard containers. They were provided with 10% sucrose solution on cotton pads and placed in iceboxes with cold packs before transport to the laboratory by car for 3 hours. They were acclimated for 2 to 6 days at 23 °C before experimental infections. Virus experiments were conducted in a biosafety level three (BSL-3) insectary at the Australian Animal Health Laboratory (AAHL). Supernatant of BTV serotype 1 (BTV-1) strain CS 156 (St George *et al.* 1980) culture was diluted in heparinised cattle blood antibody-free for BTV to a final viral titre between 3 x 10^5 and 1 x 10^6 $TCID_{50}$/ml. Sucrose *ad libitum* was stopped 24 h before infective challenge. Midges were offered an infective blood meal through one day-old chicken skin using a membrane feeding system (Hemotek®) in environmental cabinets at 15 °C, 50% relative humidity and darkness and allowed to feed for 60 min. After CO_2 anaesthesia and sorting on an entomological chill table, only blood-fed females were reserved in cardboard cups, with 10% sucrose and maintained at 23 °C in darkness for 13 day (D_{13}) extrinsic incubation for final testing. Unfed *Culicoides* were returned to containers for re-feeding on the following day. Controls for infection experiment were defined by infection of the non-vector *Culex annulirostris* colony mosquitoes challenged with the same BTV-1 strain either orally, as negative control, or by intra-thoracic microinjection of 69 nl of the undiluted virus batch corresponding to ~ 10^2 $TCID_{50}$ virus load. This micro-injection bypasses the midgut barrier and makes the mosquito able of viral infection in the hemolymph and as such, considered positive control. At D_{13}, live midges and mosquitoes were anesthetised with CO_2 and sorted on the chill table, identified at the species level (Dyce *et al.* 2007) and stored at - 80 °C in individual tubes. The infection dynamics was assessed by comparison of presence and relative quantification of viral RNA at D_0, in either *per os* or microinjection freshly challenged insects and at D_{13} in midges and mosquitoes, after extrinsic incubation time.

Viral molecular detection by qPCR followed the technique of Veronesi and colleagues (Veronesi *et al.* 2013). Briefly, samples were homogenized in 200 µl of Minimum Essential Medium for insect cell culture by bead beating at 6.0 m/s for 20 s with 10 x 1.0 mm beads. An aliquot of 100 µl was stored in - 80 °C for virus isolation. The remaining 100 µl was mixed with Magmax® nucleic acid extraction buffer for both DNA and RNA extractions. BTV-1 RNA presence and quantification was assessed by using a BTV real-time TaqMan PCR assay routinely run at AAHL and targets the NS3 gene expressed by virus genome segment 10 (Hofmann *et al.* 2008). Primers and probe were pre-mixed and stored in small aliquots at - 20 °C (Hofmann Fwd R10 189-207 5'-TGGAYAAAGCGATGTCAAA-3', Hofmann Rev R10 285-266 5'-ACATCATCACGAAACGCTTC-3', Hofmann Probe R10 245-264 5'-6FAM-ARGCTGCATTCGCAT CGTACGC-TAMRA-3'). Cycle threshold (Ct) values equal or higher than 40 were considered negative. Duplicates were required to yield the same results for a firm positive or negative conclusion. Specimens giving a positive/negative result for each duplicate were treated as inconclusive. In each qPCR run were included a strong and a weak positive controls with standard BTV RNAs, giving respective Ct values around 24 and 32, and a negative control without viral RNA. To exclude any major effect of midge materials presence on molecular detection, a dilution curve was composed of four 10 x serial dilutions of tissue culture super natant (TCSN) virus mixed with individual unfed midges. Quality of RNA extraction was internally controlled by a separate TaqMan assay targeting 18S ribosomal RNA with specimens showing higher than 25 Ct values being discarded. The mean viral RNA relative index (invdiff) was estimated with standardization after the eukayotic 18S RNA by the calculation of the multiplicative inverse of the difference between BTV and 18S Ct values ['invdiff' = 1 / (BTV Ct - 18S Ct)], (Figure 1). A subset of 31 positive RNAs, including freshly bloodfed specimens of midges and mosquitoes, were tested for another qPCR targeting BTV segment 1 (Shaw *et al.* 2007) with another set of primers (BTVrsa 291-311For 5' -GCGTTCGAAGTTTACATCAAT- 3', BTVrsa 387-357Rev 5'-CAGTCATCTCTCTAGACACTCTATAATTACG-3',Probe RSA-BTV 341-320 5' - CGGATCAAGTTCACTCCACGGT-3'). Similar to the first qPCR, strong and weak BTV RNAs positive controls, as well as a negative one and 18S were used in this PCR run. The confirmed double-positive D_{13} *Culicoides* specimens, with double-positive D_0 midges and mosquitoes as positive controls, were tested for presence of viable virus. More, egg homogenates previously injected with BTV-1 infected blood or PBS were included as supplementary, positive and negative controls, respectively. After ten day amplification culture on Kc/FLI *C. variipennis* cells (kindly provided by Istituto

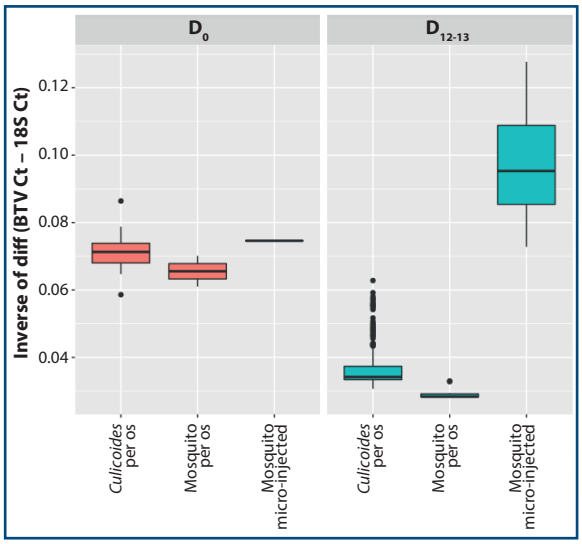

Figure 1. *Boxplots of normalized BTV RNA index standardized to the 18S ribsoomal RNA [Y-axis: invdiff = 1/(BTV Ct – 18S Ct)].* On the left side, red boxplots with values at day 0 for, from the left to the right: *per os* infected *Culicoides*, *per os* infected mosquitoes, and microinjection infected msoquitoes. On the right side, with same order, blue boxplots for day 13 values.

Zooprofilattico Sperimentale dell'Abruzzo e del Molise, IZSAM, Italy) (Wechsler *et al.* 1989), samples were tested for cytopathogenic effect (CPE) on Vero cells, and BTV antigen detection by ELISA technique following Stanilawek and colleagues (Stanilawek *et al.* 1996) and Hawkes and colleagues (Hawkes *et al.* 2000), using Nunc Maxisorp ELISA immuno-plates (Thermo Fisher Scientific™) and insect cell supernatants. The controls of the cell culture phase were used. Statistics and graphics were obtained by using R (R Core Team 2017) and ggplot2 package (Wickham 2016).

The rate of blood-feeding for *Culicoides* was 49.8% (n = 3293). The rate for mosquitoes was 90.6% (n = 53). The immediate (D_0) mortality rate was 15% for *Culicoides*. The percentage survival for *Culicoides* at D_{13} was 25.2% (n = 1641), 58.3% for bloodfed mosquitoes (n = 24), and 37.5% for microinjected mosquitoes (n = 24).

Among the 416 *Culicoides* females tested by qPCR, 94.5% were *Culicoides austropalpalis* Lee and Reye, 1.7% *Culicoides marksi* Lee and Reye, 1.2% *Culicoides victoriae* Macfie, 0.7% *Culicoides bundyensis* Lee and Reye, 0.7% *Culicoides ornatus* grp and 1.2% were not determined. The mean Ct values for TaqMan PCR assay of virus and midge serial dilution (from $TCID_{50}$ 10^1 to 10^4) were 26.8, 23.7, 20.1, and 16.6, respectively. The invdiff (Figure 1) for freshly bloodfed *Culicoides* (D_0) were 0.071 (SD = 0.007) and 0.065 for mosquitoes (SD = 0.006), and do not differ significantly (Kruskal-Wallis test p = 0.27). The D_0 value for microinjected mosquitoes was 0.074 and did not differ from *per os* orally challenged mosquito values (Kruskal-Wallis test, p = 0.22). The prevalence of positive specimens (Table I) at D_{13} was 7.4% (31/416) for *Culicoides*, 0% for bloodfed mosquitoes (0/14) and 100% (9/9) for microinjected mosquitoes.

Table I. *Prevalence of positive, negative and inconclusive Ct values for NS3 BTV-1 at RT-PCR D_{13}.*

	Positive (%)	Negative (%)	Inconclusive (%)
Culicoides blood fed (n = 416)	31 (7.4)	338 (81.3)	47 (11.3)
Mosquitoes blood fed (n = 14)	0 (0)	12 (85.7)	2 (14.3)
Mosquitoes microinjected (n = 9)	9 (100)	0 (0)	0 (0)

Of 31 NS3 qPCR positive specimens, 29 (94%) were *C. austropalpalis*, one *C. marksi* and one undetermined. The positive RNA values for these two last species were not different from those for *C. austropalpalis*. All the inconclusive samples were *C. austropalpalis*. For *Culicoides*, D_{13} average viral RNA relative values were 0.036 (SD = 0.005), with positive values at 0.052, (SD = 0.0049). Both were significantly ($p < 0.0001$) lower than D_0 values. For microinjected mosquitoes, the D_{13} average Ct values at 0.099 (SD = 0.019) was higher than D_0 but not significantly (p = 0.223). However, this D_{13} value for micro-injected mosqutioes was significantly ($p < 0.001$) higher than the per-os infected mosquito at D_{13} average value of 0.029 (SD = 0.002) (Figure 1).

Of the 29 NS3 BTV-1 positive D_{13}-infected *Culicoides austropalpalis* tested, only four were tested positive for RSA Segment 1 qPCR, six tested inconclusive and 19 were negative. As control, two D_0 infected *Culicoides* were positive. In total, approximately 1% of the challenged *Culicoides* females presented viral RNA from two different genes. None of these four specimens with viral RNA at D_{13} showed CPE on Vero cells, after amplification attempt during ten days on *Culicoides* Kc cells. As control, CPE was demonstrated in 100% of D_0-infected *Culicoides* [n = 4, average Optical Density (DO) 2.178 +/- 0.102] and 100% of mosquitoes infected at starting stage of infection (n = 9, average OD 2.229 +/- 0.57).

We performed experimental infections on midges caught at a Victorian farm of mixed cattle and sheep, and challenged more than 3,000 *Culicoides* by membrane feeding of BTV-1 infected blood meal. We obtained a feeding rate of approximately 50%, comparable to experiments conducted in Europe by Carpenter and colleagues (Carpenter *et al.* 2008) on field collected midges. Our 25% survival rate was lower than the 39% obtained by Carpenter and colleagues (Carpenter *et al.* 2008) but the incubation period was longer: 13 days instead of 7-10 days and a membrane-feeding instead of pad-feeding technique was used. The viral RNA detection method

developed by Veronesi and colleagues (Veronesi *et al.* 2013) allowed individual testing of midges with up-scaling to more than 400 midges. The dilution curve for positive controls gave consistent Ct values. Seven percent of the midges tested gave positive results for the presence of viral RNA 13 days after infective feeding. However, the invdiff were siginificantly lower than at D_0, indicating that viral replication was low or null. The *Culex annulirostris* mosquitoes used as D_0 control gave not significantly different viral RNA values, despite the probable blood meal volume difference between the two insects. However, and as expected with mosquitoes and BTV, results became negative with time after oral challenge. However, following microinjection and the midgut barrier bypass, increased viral RNA indices, indicative of viral replication, justified the mosquito model for positive control, as with other insect models (Shaw *et al.* 2012).

Differences in vector competence according to virus serotypes or strains have been described (Bellis *et al.* 1994). Our experiments were conducted to determine for vector competence for BTV-1, the most common serotype (Firth *et al.* 2017) circulating in the adjacent more northerly state of New South Wales and one of the most probable to invade southern grazing regions. However, we cannot readily extrapolate our results to other serotypes and especially exotic BTVs.

Culicoides austropalpalis is reported to be particularly abundant in eastern Australia (Kettle and Elson 1975), in south east Queensland (Wild 1984) and in Victoria (present work). However, its vector capacity for livestock pathogens is potentially compromised by its predominantly ornithophilic behaviour (Kat *et al.* 1978). Similarly, African bird feeder *Culicoides,* being found BTV positive in experimental infections (Paweska *et al.* 2002) and isolations (Nevill *et al.* 1992), have been considered of low epidemiological importance. However, feeding behaviour is rarely fully restrictive and *C. austropalpalis* has been found to also feed on cattle at a relatively high rate of 46% (Van der Saag *et al.* 2016), and also on marsupials (Kay *et al.* 1978). Detection of viral RNA of Wallal virus (an orbivirus responsible for kangaroo blindness) in *C. austropalpalis* (Hooper *et al.* 1999) suggests it may be a vector but BTV has never been isolated from pools of *C. austropalpalis* (Standfast *et al.* 1984). Therefore, despite its abundance in the farm environment in Australian temperate regions, our data and its mainly ornithophilic diet indicate that *C. austropalpalis* is of low, if not null, epidemiological importance for BTV transmission.

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