# *Evaluation of* in vitro *methods for assessment of infection of Australian* Culicoides *spp. with Bluetongue viruses*

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**Summary**
Biting midges from the genus *Culicoides* (Diptera: Ceratopogonidae) are the vectors of several globally important arboviruses that affect livestock. These include orbiviruses from the Bluetongue virus (BTV) and African horse sickness virus (AHSV) groups and members of the Simbu serogroup of orthobunyaviruses, such as the recently emerged Schmallenberg virus. In this article, the authors evaluate several methods for feeding wild-caught Australian *Culicoides* on BTV infected preparations of blood and sucrose. Feeding *Culicoides* on the membrane of embryonated chicken eggs was identified as the preferred feeding method. Although, cotton wool pads soaked in either virus-infected blood or virus-sucrose mixtures were also successful. A non-destructive nucleic acid extraction technique for the detection of viral RNA in *Culicoides* was also evaluated as it allows for readily differentiating infected from non-infected *Culicoides*.

# *Metodi di infezione* in vitro *sperimentale con il virus della Bluetongue in specie australiane di* Culicoides

**Parole chiave**
*Culicoides*,
Infezione sperimentale,
RT-PCR,
Vettore,
Virus della Bluetongue.

**Riassunto**
Il genere *Culicoides* (Diptera: Ceratopogonidae) include specie vettrici di numerosi virus (arbovirus) in grado di provocare ingenti danni al patrimonio zootecnico. Tra questi sono sicuramente da menzionare il genere degli orbivirus con i gruppi del virus della Bluetongue (BTV) e della Peste equina e quello degli orthobunyavirus con i gruppi e membri del sierogruppo Simbu come il virus dello Schmallenberg. In quest'articolo, gli autori valutano i diversi metodi di infezione sperimentale di *Culicoides* di campo con preparazioni a base di sangue e saccarosio infetto da BTV. L'infezione di *Culicoides* attraverso la membrana di uova embrionate di pollo è quella che ha dato i risultati migliori anche se l'infezione utilizzando tamponi di ovatta imbevuti di sangue infetto o miscele virus-saccarosio è stata anch'essa efficace. L'articolo analizza anche una tecnica conservativa di estrazione degli acidi nucleici per rilevare RNA virale in *Culicoides* che si è dimostrata utile per differenziare gli insetti infetti da quelli non infetti.

## Introduction

There are approximately 1,400 recognised species of *Culicoides* worldwide (Bakhoum *et al.* 2013). However, data relating to the competence of many species to transmit arboviruses such as Bluetongue virus (BTV) is very limited or incomplete. Consequently, tools and techniques to study the interaction of *Culicoides* and the viruses that they transmit has been identified as a the main area of *Culicoides* vector biology that requires investigation (Koenraadt *et al.* 2014). To achieve this, methods are required to assess *in vitro* which *Culicoides* will take a virus-infected blood meal. Recent studies have tended to favour the use of techniques such as membrane feeders (Veronesi *et al.* 2013 a, b) and cotton wool pads (Venter *et al.* 2005). These methods do not require the use of animals, avoiding ethical, husbandry, and management considerations associated with the use of live animals.

Following the recent epidemics of BTV and Schmallenberg virus (SBV) in Europe, molecular methods for the detection of viral RNA in *Culicoides* have been widely adopted as part of entomological surveillance programs (Veronesi *et al.* 2013a). A disadvantage common to all molecular methods is that they do not discriminate between infectious virus and viral RNA. The use of probe based semi-quantitative assays, like quantitative real time polymerase chain reaction (qRT-PCR), in which results are reported as cycle-threshold (Ct) values has partially helped to resolve this issue. This is because samples with high levels of viral RNA consistent with viral replication in the insect can be easily distinguished from those containing low levels of viral RNA, likely derived from a blood meal on the basis of Ct value (Hoffmann *et al.* 2009, Vanbinst *et al.* 2009).

Despite the adoption of qRT-PCR for surveillance of arboviruses in *Culicoides*, there have been very few studies to investigate the kinetics of infection via qRT-PCR. Veronesi and colleagues (Veronesi *et al.* 2013a) used a strain of BTV-1 to investigate the relationship between the quantity of viral RNA detected by qRT-PCR and presence of infectious virus in *Culicoides*. They deemed colonised *Culicoides sonorensis* to be infected if evidence of viral replication (no minimum threshold given) in a midge could be detected by qRT-PCR. This was indicated by a significantly lower Ct value in an individual midge at the end of the extrinsic incubation period (EIP) compared to a midge assayed immediately after feeding. Using this criterion, the infection rate based on qRT-PCR was equivalent to the rate determined by isolation of infectious virus (Veronesi *et al.* 2013a). Prior to this, detection of $10^{2.5\text{-}3.0}$ 50% tissue culture infectious dose ($TCID_{50}$) of BTV (regardless of strain) in *C. sonorensis* was considered to be indicative of viral infection (Mellor 2000).

The primary objective of the experiments described in this study was to evaluate 3 methods for feeding wild caught species of Australian *Culicoides* on BTV infected preparations. These were the use of an artificial membrane feeder, direct feeding from cotton wool pads, and feeding on embryonated chicken eggs (ECEs). The secondary objective of this study was to evaluate a non-destructive digestion and nucleic acid purification technique (Bellis *et al.* 2013) for the detection of BTV RNA in *Culicoides* after feeding on virus infected preparations.

## Materials and methods

### Defibrinated bovine blood

Blood (500 ml) was collected from a single Holstein-Friesian steer that was free from BTV (RNA and antibody, data not shown) via jugular venipuncture (12 g needle). The blood was collected into a sterilised side arm flask (1 l) containing 3 layers of glass beads (6 mm diameter). As the blood began to cover the beads, a gradual swirling motion of the beads was begun and continued until defibrination of the blood was complete. This was indicated by a change in pitch when swirling the beads and formation of white foam (fibrin) on top of the blood. Once complete, the defibrinated blood was poured off from the side arm flask into sterile screw-top containers (50 ml) through sterile gauze and a glass funnel.

### Viruses

Cell culture adapted BTV serotypes 1, 2, and 23 were obtained from the Virology Laboratory, Elizabeth Macarthur Agriculture Institute, New South Wales, Australia. Serotype 1 was isolated from a blood sample from sentinel cattle in the NT, inoculated into ECEs, passaged once in *Aedes albopictus* cells (C6/36), twice in the BHK derived cell line BSR and then at least 3 times in hamster lung (HmLu-1) cell culture. Serotype 2 was a virus originally isolated from a blood sample from sentinel cattle in South-East Queensland, inoculated into ECEs, passaged once in *Aedes albopictus* cells (C6/36), twice in the BHK derived cell line BSR, and then 3 times in HmLu-1 cell culture. Serotype 23 isolated from a blood sample from sentinel cattle in the Northern Territory (NT), inoculated into ECEs, passaged once in *Aedes albopictus* cells (C6/36), twice in the BHK derived cell line BSR, and then at least 3 times in HmLu-1 cell culture. The titres (in HmLu-1 cells) of the stock virus solutions were $10^{4.86}$, $10^{4.73}$, and $10^{6.04}$ $TCID_{50}$/ml

for BTV seroyptes 1, 2, and 23, respectively. Wild type BTV serotype 1 (V8837) was provided by Dr Lorna Melville, Berrimah Veterinary Laboratory (BVL), Darwin, Australia. This was in the form of a blood sample from a sentinel steer, collected at Beatrice Hill Research Farm, Darwin, Australia, in October, 2012. The sample was strongly reactive in the pan-reactive BTV qRT-PCR (Gu *et al.* 2014) with a Ct value of 24.5 and confirmed as BTV-1 in a type-specific qRT-PCR assay (data not shown).

## Insects

Experiments described in this paper were performed on *Culicoides* collected from New South Wales (NSW) and the Northern Territory (NT), Australia. A single species, *Culicoides brevitarsis*, the most widely distributed vector midge species in Australia for viruses in the *Reoviridae* and *Bunyaviridae* families, was obtained from NSW. *Culicoides brevitarsis* were reared from bovine dung collected from Duck Creek, Ballina (28°52'S, 153°30'E) and CB Alexander Agricultural College, Tocal, Paterson (32°37'S, 151°35'E). Dung of the appropriate age for the emergence of adult *C. brevitarsis* (2-3 week old) was collected from paddocks where cattle had grazed 7-21 days previously. Dung was placed in emergence chambers as described by Nicholas and McCorkell (Nicholas and McCorkell 2014) and kept at 28°C and 85% relative humidity (RH). Insects that emerged from dung and into the collection chamber were collected daily. *Culicoides brevitarsis* were sorted from the other insects and placed in holding containers, such that only nulliparous *C. brevitarsis* were used in the experiments.

*Culicoides* were also collected from Northern Australia at Beatrice Hill Farm, 50 km South-East of Darwin on the Adelaide River flood plain (12°39'S 131°20'E) in October 2012 (Trials 4 and 6, Table I). Four species of *Culicoides* were collected, namely *Culicoides actoni*, *C. brevitarsis*, *Culicoides peregrinus*, and *Culicoides marksi*. Insects were collected using an aspirator (petrol powered vacuum system) from open pens of 3 to 6 cattle, for 5 minutes every 30 minutes. Insects were transported to BVL in a polystyrene box, anaesthetised with $CO_2$ and sorted to *Culicoides* genus level on a chilled surface under a stereo-microscope. Only strong, viable and nulliparous insects were selected for these studies. The *Culicoides* were not sorted to species level prior to feeding experiments as it was not possible to morphologically identify individual *Culicoides* on the chill table at a rate fast enough to ensure their survival after chilling.

All *Culicoides* were placed in holding containers that consisted of a cardboard cup (12 oz) with a fine cloth screen secured by 2 rubber bands. The *Culicoides* were incubated at 28°C for 2-3 days and provided a 10% sucrose solution twice daily. *Culicoides* were starved for 12-24 hours before experimental feeding attempts. After a feeding period of up to 12 hours, all insects were examined and sorted on the basis of the presence of blood or coloured virus-sucrose mixture in the abdomen.

## Methods of feeding *Culicoides*

### *Artificial membrane feeding – uninfected blood (Trial 1)*

A total of 881 *C. brevitarsis* from NSW were placed on a membrane feeding machine in batches of 50-300 insects (Hemotek Ltd, Blackburn, UK) containing

**Table I.** *Evaluation of feeding rate of* Culicoides *using different feeding methods.*

| Trial | Method of feeding | Type of meal (and serotype) | Species of *Culicoides* | Total number *Culicoides* exposed to treatment | Total number of fed females (Proportion) |
|---|---|---|---|---|---|
| 1 | Membrane feeder A | Uninfected blood | *C. brevitarsis* | 367 | 0 (0%) |
| | Membrane feeder B | Uninfected blood | *C. brevitarsis* | 444 | 0 (0%) |
| 2 | Cotton wool pad | Uninfected blood | *C. brevitarsis* | 4,167 | 373 (9%) |
| 3 | Cotton wool pad | Tissue culture supernatant-sucrose mixture (BTV 1, 2 and 21) | *C. brevitarsis* | 3,608 | 2,634 (73%) |
| 4 | Cotton wool pad | Wild type virus-sucrose mixture (BTV-1) | *C. actoni*<br>*C. brevitarsis*<br>*C. marksi*<br>*C. peregrinus* | 1,766 | 654 (37%) |
| 5 | ECE | Uninfected ECE | *C. brevitarsis* | 464 | 54 (11%) |
| 6 | ECE | Infected with wild type virus (BTV-1) | *C. actoni*<br>*C. brevitarsis*<br>*C. marksi*<br>*C. peregrinus* | 540 | 300 (55%) |

uninfected defibrinated bovine blood for 12 hours to investigate whether they would feed. The blood was maintained at 37°C in the feeder. Two types of membranes were evaluated. The first was Parafilm M® (Bemis NA, Neenah, WI, USA), stretched as thin as possible (membrane feeder A, Table I) and the second was a collagen membrane (Discovery Workshops, Accrington, UK) (membrane feeder B, Table I). After feeding, *Culicoides* were anaesthetised with $CO_2$ and preserved in 70% ethanol. The proportion that fed was then calculated based on the presence/absence of blood in the abdomen.

### *Cotton wool feeding*

#### *a) Uninfected blood (Trial 2)*

Replicates of 50-300 *C. brevitarsis* from NSW (a total of 4,167) were fed on cotton wool pads saturated in pre-warmed (37°C) uninfected defibrinated bovine blood and placed on the top of the mesh of the holding containers following the method described by Venter and colleagues (Venter *et al.* 2005). *Culicoides* were allowed to feed for up to 12 hours at 28°C. Time constraints precluded counting of identical numbers of *C. brevitarsis* into each replicate. After feeding, *Culicoides* were anaesthetised with $CO_2$ and preserved in 70% ethanol. The proportion that fed was then calculated on the basis of the presence/absence of blood in the abdomen.

#### *b) Virus in sucrose (Trial 3)*

Separate groups of *C. brevitarsis* from NSW were offered sucrose solutions spiked with cell culture adapted BTV serotypes 1, 2, and 23. The tissue culture supernatant virus solutions were mixed with 30% sucrose in the ratio of 2/3$^{rd}$ virus solution to 1/3$^{rd}$ sucrose, giving a final concentration of 10% (w/v). Bluetongue virus RNA concentration as determined by qRT-PCR for each virus-sucrose solutions fed to *Culicoides* was 16.7, 14.2 and 14.6 for serotypes 1, 2 and 23, respectively. Each of the virus-sucrose solutions was coloured with 0.2% (v/v) green food dye (Queen fine foods, Queensland, Australia) as previously described by Muller (Muller 1985). *Culicoides* were fed on the virus-sucrose mixtures as described in previously. After feeding, *Culicoides* were sorted microscopically into fed and unfed on the basis of the presence of colour in the abdomen and placed in new holding containers. Up to 10 *Culicoides* that had engorged on virus-sucrose mixtures were tested by qRT-PCR immediately after feeding. The remaining fed *Culicoides* were held at 28°C and were given access to a 10% (w/v) sucrose solution daily. Live *Culicoides* (n = 12-97) were collected at various time intervals during the EIP. The number collected and the frequency of collection depended on the proportion of *Culicoides* that survived. The *Culicoides* were stored in 70% ethanol and then subjected to analysis by qRT-PCR.

#### *c) Infected blood in sucrose (Trial 4)*

A mixture of *Culicoides* species collected in the NT was fed wild type BTV serotype 1 (V8837) as a blood-sucrose mixture via cotton wool pads. The bovine blood containing infectious BTV (Ct value 24.5) was mixed to achieve a final sucrose concentration of 10% and warmed to 37°C. After feeding on the cotton wool pads for 12 hours at 28°C, *Culicoides* were sorted into fed or unfed. Fed insects were incubated at 28°C and 85% RH and were given a 10% sucrose solution twice daily for the putative EIP of 9-10 days. Midge-holding containers were inspected for any dead *Culicoides* each day and these were discarded. Fed *Culicoides* which survived the EIP were tested individually for the presence of BTV RNA.

### *ECE feeding (Trials 5 and 6)*

Four species of *Culicoides* from the NT and 1 species from NSW were fed on ECEs using a modified version of a previously published method (Jones and Foster 1966, Foster and Jones 1973, Boorman *et al.* 1975). Embryonated chicken eggs were obtained from a commercial supplier and were 13 days of age at time of feeding. Both infected and uninfected ECEs were used. The modified method used was as follows. Firstly, part of the shell over the air sac was removed with a small electric rotary cutting tool. The shell membrane was then dampened with phosphate buffered saline solution (pH 7.3). A small cylindrical plastic specimen container (25 ml), to which *Culicoides* were later added, was affixed to the egg using masking tape. Viable *Culicoides* were introduced to the plastic specimen container via a small port sealed with rubber dental dam (Henry Schein Halas, Waterloo, NSW, Australia). *Culicoides* were allowed to feed for 12 hours during which time the eggs were incubated at 32°C.

Uninfected ECEs were used to perform an initial evaluation of the ECE feeding technique (Trial 5). Nine groups of *C. brevitarsis* (n ~ 50) from NSW were given access to ECEs prepared as described above. After feeding, blood fed *Culicoides* were sorted from non-blood fed *Culicoides* on a chill table, and the proportion of blood fed *C. brevitarsis* was calculated. No attempt was made to estimate survival rate of the insects after feeding since they were all preserved in 70% ETOH immediately after the feeding attempt.

In Trial 6, *C. actoni*, *C. brevitarsis*, *C. peregrinus*, and *C. marksi* from the NT were fed on virus infected

ECEs. The ECEs were inoculated at 11 days of age via the intravascular route with 50 µl of blood containing wild type BTV serotype 1 (V8837) that had been diluted 1/10 in sterile water. Nulliparous *Culicoides* of mixed species composition were given access to the membrane of BTV infected ECEs 36 hours after inoculation, as described above. After feeding, the insects were anaesthetised with $CO_2$ and sorted microscopically (engorged/non-engorged) on a chill table. Blood fed *Culicoides* were placed in a holding container for a putative EIP of 9 days at 28°C, 85% relative humidity and given a 10% sucrose solution twice daily. Midge-holding containers were checked for dead *Culicoides* daily and these were discarded.

### Enzymatic digestion: individual insects

Non-destructive digestion was undertaken on all surviving insects from the experiments in which insects were fed on virus (Trials 3, 4, and 6). The enzymatic digestion method was undertaken as described by Bellis and colleagues (Bellis *et al.* 2013) with minor modifications. Briefly, individual *Culicoides* were placed in separate wells of a 96 deep well plate (Cat. No. 97040450, ThermoScientific, Waltham, MA, USA). A tissue digest solution consisting of 1 part Digest enzyme (DX) (Cat. No. 950120, QIAGEN, Limburg, Netherlands) to 99 parts Tissue digest reagent (DXT) (Cat. No. 950183, QIAGEN, Limburg, Netherlands) was prepared and 100 µl added to each well containing a midge. The plate was then sealed and incubated at 37°C overnight on a plate shaker which was moving gently. After 12-16 hours, 50 µl of digest solution was taken from each well, taking care not to remove or damage the midge. Total nucleic acid was purified from each midge digest as described below. Excess digestion fluid was removed and stored at -20°C in 1.5 ml microcentrifuge tubes. The bodies of the *Culicoides* were preserved by adding 200 µl of 70% ethanol and the insects were carefully transferred to a new 96 well plate and stored at +4°C.

### Nucleic acid extraction

Total nucleic acid was purified from 50 µl insect digest fluid using the MagMAX-96 Viral RNA isolation kit (Cat. No. AM 1836-5, Ambion, Texas, USA) on a Kingfisher™ 96 magnetic particle handling system (KF-96 Thermo Fisher Scientific, Vantaa, Finland) as described by Gu and colleagues (Gu *et al.* 2014). Nucleic acid was eluted in a 50 µl volume and either tested immediately or stored at -20°C in 96 well elution plates sealed with adhesive foil. In each nucleic acid extraction run, 2 positive controls (Ct value of 28 and 31) and 1 negative control (tRNA) were included.

### qRT-PCR parameters

The RNA of BTV was detected by qRT-PCR using the pan-BTV reactive assay (Hofmann *et al.* 2008b) with mastermix, controls, cycling conditions, and sample volumes as described by Gu and colleagues (Gu *et al.* 2014). Due to the exponential nature of qRT-PCR in an optimised assay a 10-fold change in viral RNA concentration is equivalent to a change in approximately 3.3 Ct units. Threshold cycle values ≥ 38 were considered inconclusive and ≥ 40 were considered negative.

## Results

The proportion of *Culicoides* that fed using different feeding systems and meals is shown in Table I. In Trial 1, no *C. brevitarsis* could be induced to feed through the artificial membrane feeder, with either a parafilm or collagen membrane. Insects fed successfully on cotton wool pads and ECEs (Trials 2-6). A low proportion of *C. brevitarsis* fed on undiluted blood from cotton wool pads in Trial 2. For each of the trials using the cotton wool pad method, there was a significant difference ($P<0.05$) in the proportion of *Culicoides* that fed and considerable variation between replicates for within a trial.

In Trial 3, *C. brevitarsis* were fed on separate solutions of cell culture adapted BTV strains mixed with sucrose (Table II). Colouring of the feeding solution with food dye readily enabled the identification of *Culicoides* that had fed. Viral RNA was detected at low levels in *C. brevitarsis* up to 22 days (BTV-1 RNA) probably indicating residual RNA from the original blood meal (see Discussion section). The mean Ct value of BTV RNA detected in *Culicoides* immediately after feeding was 33.9, 34.8, and 31.8 for BTV-1, BTV-2, and BTV-23, respectively. This was reflective of the titre of the original virus solutions. For example, BTV-23 was approximately 10 fold more concentrated than BTV-1 and BTV-2, and this was reflected in the Ct value of *C. brevitarsis* assayed after feeding. Viral RNA could be detected in 73-100% of *C. brevitarsis* immediately after feeding. One midge had a markedly higher concentration of BTV-1 RNA 17 days after feeding, with a Ct value 21.4. This is a difference of 12 Ct values compared to the mean Ct value of *Culicoides* assayed immediately after feeding and possibly indicates a possible increase of $>10^{3.5}$ in viral RNA concentration. There was no evidence of significant viral replication in any *C. brevitarsis* fed on virus-sucrose mixtures of BTV-2 or BTV-23, as indicated by high Ct values.

Four species of *Culicoides* were collected in the NT and fed using 2 methods, namely cotton wool pads saturated with a mixture of wild type BTV-1 and sucrose (Trial 4) and ECEs infected with wild type

**Table II.** *Number positive and concentration of viral RNA in* Culicoides brevitarsis *at various intervals after feeding from cotton wool pads containing cell culture adapted Bluetongue virus serotypes 1, 2 and 23, respectively.* Ct values in bold indicate evidence of virus replication in the insect.

| Virus | Days post feeding | Number positive/ number tested (%) | Average Ct value | Range of Ct values |
|---|---|---|---|---|
| BTV-1 | 1 | 74/97 (76) | 33.85 | 29.08-37.31 |
| | 2 | 17/26 (65) | 35.03 | 32.80-37.24 |
| | 3 | 28/33 (85) | 34.62 | 32.29-38.36 |
| | 4 | 19/41 (46) | 35.25 | 33.63-37.70 |
| | 5 | 14/34 (41) | 34.56 | 32.48-36.10 |
| | 6 | 9/16 (56) | 34.00 | 29.88-35.58 |
| | 7 | 8/14 (57) | 34.39 | 30.57-37.79 |
| | 8 | 3/12 (25) | 36.31 | 35.67-36.63 |
| | 10 | 17/48 (35) | 35.45 | 32.30-38.10 |
| | 11 | 4/19 (21) | 36.34 | 35.87-36.60 |
| | 12 | 15/28 (54) | 35.81 | 33.76-37.12 |
| | 17 | 12/94 (13) | 34.83 | **21.43**-39.68 |
| | 18 | 3/28 (11) | 36.23 | 35.40-36.95 |
| | 20 | 3/32 (10) | 34.22 | 31.47-36.56 |
| | 22 | 14/68 (21) | 35.24 | 31.03-36.80 |
| BTV-2 | 1 | 8/11 (73) | 34.82 | 31.85-37.10 |
| | 14 | 9/78 (12) | 36.54 | 35.66-38.03 |
| | 21 | 3/26 (12) | 36.62 | 34.40-37.80 |
| | 24 | 6/26 (23) | 35.87 | 32.80-37.26 |
| BTV-23 | 1 | 17/17 (100) | 31.78 | 28.53-33.67 |
| | 10 | 56/82 (68) | 34.90 | 32.79-36.85 |
| | 14 | 4/12 (33) | 34.64 | 33.79-35.38 |
| | 16 | 16/38 (42) | 35.69 | 30.10-38.25 |
| | 17 | 10/29 (35) | 33.64 | 29.54-36.77 |

BTV-1 (Trial 6, Table I). The predominant species in the collection was the recognised vector species *C. actoni* (Standfast *et al.* 1985). Small numbers of another vector species *C. brevitarsis* (Standfast *et al.* 1985) and the non-vector species *C. marksi* and *C. peregrinus* (Standfast *et al.* 1985) were also collected. The proportion of *Culicoides* that took a blood meal from the cotton wool pads in Trial 4 was 37%. The survival of these *Culicoides* during the putative EIP of 9-10 days was 55%, which included 204 *C. actoni*, 16 *C. brevitarsis*, 136 *C. marksi*, and 1 *C. peregrinus*. When each of the surviving *Culicoides* was assayed individually, low levels of BTV RNA (high Ct values) were detected in a small proportion of *Culicoides* [7.8% of *C. actoni* (average Ct value 35.4, range 32.4-37.6) and 7.4% of *C. marksi* (average Ct value 35.8, range 34.7-36.7)]. However, there was no evidence of any individual insect in which viral replication had apparently occurred. The proportion of *Culicoides* that fed in Trial 4 varied from less than 20% for one replicate to a maximum of 70% for another.

The ECE feeding method was attempted with both uninfected (Trial 5) and ECEs infected with wild type BTV-1 (Trial 6). In the absence of virus, a feeding rate of 11% was obtained for *C. brevitarsis* (Table I). When mixed species of *Culicoides* in the NT were fed on wild type BTV-1 infected ECEs in Trial 6, a total of 300 (55%) took a blood meal. The survival rate of these *Culicoides* was 51% (153/300) over a putative EIP of 9 days. The surviving insects – consisting of 122 *C. actoni*, 7 *C. brevitarsis*, 22 *C. marksi*, and 1 *C. peregrinus* – were tested for BTV individually. Significant viral replication was evident in 3/122 (2.5%) of *C. actoni* that were tested. The Ct values of these individual insects were 19.14, 19.43, and 23.7, respectively. The average Ct value of blood taken from the ECEs at the time of feeding was 31.75 per µl. Immediately after feeding, small numbers of *C. actoni* were assayed (average Ct value 34.3, range 29.5-36.5). An additional 13 *C. actoni* tested positive after the EIP (average Ct 34.5, range 29.3-36.4), which is not different to the Ct value of an insect assayed immediately after feeding. A greater proportion of *Culicoides* fed on ECEs inoculated with virus compared to un-infected ECEs ($P < 0.01$). However, there was systematic variation in the proportion of *Culicoides* that fed in each of the replicates of the two trials in which the ECE method was used.

## Discussion

The primary objective of this study was to evaluate several artificial or laboratory blood feeding methods on wild caught Australian *Culicoides*. Prior to this study, the only method successfully employed for vector competence studies of *Culicoides* in Australia involved feeding on virus infected animals (Muller 1979, Muller 1985). The experiments in this study were undertaken with the aim of identifying a successful artificial feeding method so that vector competence studies could then be instigated. Of the 3 methods that were evaluated, *Culicoides* could be induced to take a blood meal from cotton wool pads and ECEs when virus was both present or absent.

None of the *Culicoides* fed from the artificial membrane feeder with either a parafilm or collagen membrane. Despite this, membrane feeders have been used successfully with colonised *Culicoides sonorensis* (which is a much bigger and more robust species than *C. brevitarsis*) in the US and the United Kingdom (Jones and Foster 1974, Veronesi *et al.* 2013a) and with some wild caught species in South Africa (Venter *et al.* 2011). While other membranes such as chicken skin (Venter *et al.* 2006, Del Rio Lopez *et al.* 2011) or embryonic membranes may have warranted further investigations with the membrane feeding equipment, embryonic

membranes were found to be the optimal method when used *in situ* in ECEs and offers additional benefits as described below.

Two trials were undertaken using the cotton wool pad method with either a combination of virus (tissue culture supernatant) and sucrose or naturally infected bovine blood and sucrose in an attempt to infect *Culicoides* with both laboratory adapted and wild type strains of BTV. For each of the trials in which *Culicoides* were fed on preparations containing virus, a putative 9-10 EIP was selected based on previous studies of viral replication in *Culicoides* (Standfast *et al.* 1985, Mellor *et al.* 2000, Tabachnick 2004, Veronesi *et al.* 2013a). These studies have shown that 9-10 days are an adequate span of time for susceptible *Culicoides* to develop a fully disseminated viral infection at a temperature similar to what was used in this study.

The cotton wool pad method was successful for feeding and the infection of *C. brevitarsis* with a laboratory adapted strain of BTV serotype 1 in the current study. This feeding technique was described and validated by Venter and colleagues (Venter *et al.* 2005) as an alternative means of assessing the infection rates with *Culicoides* species that are refractory to membrane feeding in the laboratory. The method has also been used to evaluate the infection of *Culicoides* in the United Kingdom for BTV-8 (Carpenter *et al.* 2006, Carpenter *et al.* 2008). Initially, the feeding rates of *C. brevitarsis* using this method with uninfected defibrinated blood were very low. However, in subsequent experiments using mixtures of virus and sucrose or blood (containing wild type BTV-1) and sucrose, the feeding rates improved significantly. Therefore, the composition and viscosity of the feeding solution may have influenced the feeding behaviour of *Culicoides* on cotton wool pads, with a decreasing concentration of blood associated with increased feeding rates.

Evidence of viral replication was detected in a single *C. brevitarsis* that fed on a mixture of cell culture adapted type 1 virus and sucrose from a cotton wool pad with an increase of at least $10^{3.5}$ fold in the concentration of viral RNA. There was no evidence of any viral replication in *Culicoides* from the NT that were fed a mixture of wild type virus and sucrose via cotton wool pads. This may have been due to a low titre of infectious virus in the blood sample or uptake of a smaller blood meal volume that may occur when the cotton wool pad technique is used, as it has been previously reported (Venter *et al.* 2005). Furthermore, there are 2 additional limitations of the cotton wool pad method that must be considered. Firstly, *Culicoides* do not have to pierce a membrane or blood vessel in order to feed. Secondly, the presence of sucrose in Trials 3 and 4 may have prevented virus from reaching the mid-gut, where the blood meal is usually held. This is because *Culicoides* have gut-diverticula that are used for the storage of carbohydrates, such as sugars (Mellor 2000). Therefore, the mid-gut of the insects may not have been exposed to the full dose of ingested virus, which may have adversely affected estimates of infection.

When *Culicoides* in the NT were fed on ECEs inoculated with a wild type BTV-1, extensive viral replication was observed in 3 *C. actoni* specimens after a putative EIP of 9 days. When compared to the average quantity of virus in the bloodmeal immediately after feeding, there was a reduction in Ct values of 10-15 units in these 3 insects, equivalent to a minimum increase of $10^3$-$10^4$ fold in the concentration of viral RNA, giving an infection rate of 2.5% (3/122) of *C. actoni* in this study. This is similar to previous reports for the infection of *C. actoni* with BTV, with estimates of 1-2% (Standfast *et al.* 1979, Standfast *et al.* 1985).

Compared to the other methods, several advantages of the ECE method were identified as part of this study. These included the ability to successfully feed a high proportion of wild caught *Culicoides* (4 species), to offer blood meals that contained a very high virus titre, and the capacity to infect ECEs with most wild strains of BTV and a range of other arboviruses. Therefore, vector competence studies with a variety of vector/virus combinations could be performed using this method. Whilst transmission of BTV by *Culicoides* to an uninfected 'clean' ECE was not attempted as part of this study, it has been successfully undertaken with *C. brevitarsis* (data not shown, manuscript in preparation). This is a substantial advantage of the ECE method, as many vector competence studies of *Culicoides* choose to imply vector competence on the basis of the detection of a threshold level of infectious virus or viral RNA, without associated transmission tests to hosts or suitable alternatives. Interestingly, there was a significant difference in the proportion of *Culicoides* that fed on infected vs. uninfected ECEs. This could have been due to the presence of virus or use of different *Culicoides* species since a mixture of species (including *C. brevitarsis*) was used in Trial 6, whereas only *C. brevitarsis* were used in Trial 5. This warrants further investigation in *Culicoides* as it has been shown previously that virus infected hosts (chickens) are more attractive to mosquito vectors (Mahon and Gibbs 1982).

In this study, high throughput molecular methods (qRT-PCR) were employed as the only method for the detection and semi-quantification of viral RNA in individual midges. The criterion for classification of a midge as infected was based on the current knowledge of viral replication kinetics in *Culicoides* (Veronesi *et al.* 2013 a, b). In this study, an infected

insect could be distinguished from a non-infected insect on the basis of the detection of significant viral replication (Veronesi *et al.* 2013 a, b). All of the insects in which significant viral replication had occurred were detected with a minimum $10^3$ fold increase in viral RNA concentration. This level of viral replication was therefore assigned as the threshold for which *Culicoides* were considered infected. In this study, lower levels of viral RNA (high Ct values) were frequently detected in a small proportion of *Culicoides*, this is most likely due to remnant RNA from the original blood meal or a limited infection of the mid-gut cells (Veronesi *et al.* 2013 a, b).

No attempt was made to isolate infectious virus because the method of nucleic extraction rendered the sample unsuitable for this. However, the non-destructive nucleic acid extraction method retains the exoskeleton of the midge in a condition that is suitable for morphological examination. This means that large numbers of mixed species of *Culicoides* can be screened for their ability to become infected using the ECE method as described above. At the same time, a detailed morphological identification (such as mounting on slides) can be undertaken on any insects in which significant viral replication is detected. In addition, all necessary epidemiological investigations – such as typing of virus strain – can now be performed with molecular tools.

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