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Assessment of the bluetongue virus vector potential of selected Culicoides species in southern Australia

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Abstract

Bluetongue virus (BTV) can cause catastrophic losses in sheep and goat populations. It is endemic in cattle across much of northern and eastern Australia with this distribution leaving the temperate zones free of virus. In 2006, the European livestock industry experienced a massive BTV outbreak occurring in previously free zones. To asses the risk of BTV outbreak in temperate zones of Australia, we have sampled *Culicoides* in various environments but focusing in cattle and sheep Victorian farms. The *Culicoides* distribution and taxonomy was updated, highlighting new cryptic species and minor incursions of specimens from neighbouring states. With a novel baiting method and molecular blood meal analysis, the feeding behaviour of endemic species was found oriented towards marsupials and birds but do not exclude bites on cattle and sheep. Quantitative PCRs run on *C. austropalpalis* individuals experimentally challenged against BTV serotype 1 did not find any viral RNA replication. The risk analysis performed for the main *Culicoides* species shows a low or null risk. The evidence of cryptic species has limited the analysis depth for these groups. However our data have restricted the gap in the knowledge hampering the understanding and capacities in mitigation for BTV incursion.

Executive Summary

Why the work was done

Bluetongue virus (BTV) is endemic in cattle across much of northern and eastern Australia. Its distribution fits well with the distribution of the major vector, *Culicoides brevitarsis*. The BTV serotypes currently present in Australia circulate mainly in cattle which serve as a reservoir of infection, and in which there has been no evidence of disease. However, Australian BTV serotypes have been shown experimentally to cause disease when sheep are exposed to them. The export trade in live animals is facilitated by the National Arbovirus Monitoring Program (NAMP) which monitors and maps the distribution of arthropod-borne viruses (including BTV) and their vectors in Australia. 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. Bluetongue can cause catastrophic losses in naïve sheep and goat populations. The duration of any disruption to trade cannot be predicted as it would be dependent upon the duration of any disease outbreak and the response of trading partners to the outbreak and control activities. $5.6 billion of (cattle, sheep & goat) meat exports would be at risk per annum plus $1 billion in live animal exports. Mortality rates in susceptible flocks can reach 30 -100%.

This project addresses one of the most critical of knowledge gaps – the identification, characterisation and assessment of risks associated with potential *Culicoides* vectors of BTV in southern Australia. We focus on the distribution of the *Culicoides* species in Victoria, their relationship with cattle and sheep, and their potential vector competence for Australian BTV serotypes.

How it was done

We collected *Culicoides* from a wide range of sites, focusing particularly in farms in cattle and sheep grazing regions of Victoria. We standardized our trapping methods for better comparison. *Culicoides* species were determined based on morphology complemented by molecular genotyping of doubtful specimens. To address the important question of host-feeding, we developed a novel approach of baiting traps with animal urine, offering the insects a dual choice for odours in the field. To complement this approach, molecular analysis of blood-fed females was performed to determine the origin of the blood taken. A risk analysis of the most abundant *Culicoides* species was conducted and compared to northern species known as vectors.

What was achieved

An up-dated revision of the *Culicoides* species in Victoria has been drawn. We have defined the distribution of the species, the incursion of new species from neighbouring states, and the main traits of biology, ecology and populations dynamics of the major species. We have classified our sites according to the abundance and the richness of the *Culicoides* species diversity. We have found hot spots where the *Culicoides* are abundant and diverse. Our genotyping helped us to show cryptic species in groups of local *Culicoides* and to begin to decipher the complex taxonomy of these groups. We have shown that the local *Culicoides* would feed preferentially on marsupials and birds, are not intrinsically particularly attracted by cattle and sheep and that only some species could feed on cattle and sheep. The most abundant species found in farms were challenged with virus and did not show any viral RNA replication by qPCR. The risk anaylsis performed shows that most of the species caught in Victoria present a low if not zero risk. However the evidence of cryptic species groups has restricted the depth of the analysis for some groups.

What industry benefits will arise from the work

We have shown that the *Culicoides* fauna in Victoria is very different form the northern zones, in its pattern (the species present, their aboundances) but also in its nature (origin and biology, including the feeding behaviour). This could explain more clearly how the current situation facing the bluetongue virus risk is set in the southern and temperate zones of Australia. We have defined the gaps in our knowledge which could limit better understanding, capacities in forecasting and mitigation of any future incursion of bluetongue virus. This places the Australian red meat industry in a much better place than was its counterpart in Europe just before the massive bluetongue serotype 8 epizootic in 2006.

Table of Contents

1 Background 8
1.1 Bluetongue virus in Australia 8
1.1.1 What is known 8
1.2 Bluetongue virus in Europe 8
1.3 Trade impact and gaps in knowledge 8
2 Projective objectives 9
2.1 Objectives 9
2.1.1 Survey of *Culicoides* species in Victoria, particularly those that occur in grazing country and are associated with cattle and sheep 9
2.1.2 Determine the host feeding preference of potential vectors 9
2.1.3 Determine the competence of potential vector species for transmission of Australian BTV serotypes 9
2.1.4 Assess the risks of BTV transmission in southern Australia 9
2.2 Outputs 9
2.2.1 By May 2013 9
2.2.2 By June 2014 9
2.2.3 By June 2015 10
2.3 Outcomes 10
3 Methodology 10
3.1 Sampling of *Culicoides* species in the field 10
3.1.1 Sampling zones 10
3.1.2 Traps 10
3.2 Identification of *Culicoides* specimens 10
3.2.1 Morphological identification 10
3.2.2 Age grading 11
3.2.3 Molecular identification 11
3.2.4 Ecological analysis 12
3.3 Host-feeding preferences 12
3.3.1 Aspiration around animals 13
3.3.2 Baited traps 13
3.3.3 Blood meal molecular analysis 13
3.4 Vector competence 13
3.4.1 Midge collections 13
3.4.2 Virus infection 14

3.4.3 Virus assessment 14
3.5 Risk analysis 15
3.5.1 Elements 15
3.5.2 *Culicoides* species 15
4 Results 15
4.1 *Culicoides* sampled in Victoria 15
4.1.1 Traps yields 15
4.1.2 Species present 15
4.1.3 Abundances 16
4.1.4 Age grading 17
4.1.5 Ecology 17
4.2 Molecular identification 19
4.3 Host preferences 21
4.3.1 Urine-baited traps 21
4.3.2 Blood meal molecular analysis 22
4.4 Vector competence 23
4.4.1 Blood-feeding and survival rate 23
4.4.2 Quantitative PCR results 23
4.5 Risk analysis 24
4.5.1 Parameters underlining vector capacity 24
4.5.2 Vector status 25
5 Discussion 26
5.1 Inferences and insights of the data 26
5.1.1 *Culicoides* in Victoria 26
5.1.2 Host preferences 27
5.1.3 Vector competence 28
5.1.4 Risk analysis 29
5.2 Practical implications for industry 30
5.2.1 Is the sheep industry fully safe in Victoria? 30
5.3 Unanswered questions / additional research recommended 31
5.3.1 Taxonomy and vector competence of *C. victoriae* complex 31
5.3.2 Trophic deviation 31
5.3.3 Extrapolation to other Australian states 31
5.3.4 Bluetongue virus serotypes 31
5.3.5 Population genetics of *Culicoides* species. Endemic species. 32
5.4 What could have been improved in the project delivery 32

5.4.1 Sampling in the field 32
5.4.2 Human ressources 32
5.4.3 Links with professional associations 33
5.5 Meeting the specific objectives of the project 33
5.5.1 Survey of *Culicoides* species in Victoria, particularly those that occur in grazing country and are associated with cattle and sheep 33
5.5.2 Determine the host feeding preference of potential vectors 33
5.5.3 Determine the competence of potential vector species for transmission of Australian BTV serotypes 33
5.5.4 Assess the risks of BTV transmission in southern Australia 33
5.5.5 Success in achieving milestones 33
6 Conclusions/recommendations 35
6.1 Future R&D 35
6.1.1 Research on Southern *Culicoides* 35
6.1.2 Research on virus 35
6.1.3 Farm environment 35
6.2 Practical activities 35
6.2.1 Review of NAMP sampling sites in Victoria 35
7 Key messages 35
7.1 The situation of the temperate zones of Australia is very particular 35
7.1.1 Beyond the climate 35
7.1.2 The vectors and their hosts 36
7.1.3 The risk 36
8 Bibliography 36
9 Appendix 37
9.1 Data tables 37
9.1.1 List of taxa collected during our sampling 37
9.1.2 Ecological indices by site 39
9.1.3 Average mean *Culicoides* caught by Green LED traps by site in Victoria (NT's site sin bold as a control) 40
9.1.4 Distribution of species 41
9.1.5 Abdomen status by species in NT and Victoria 42

1 Background

1.1 Bluetongue virus in Australia

1.1.1 What is known

Bluetongue is a viral infection of sheep, cattle and other ruminants that is transmitted by biting midges (*Culicoides* spp.). Cattle usually serve as asymptomatic reservoirs of bluetongue infection with disease occurring primarily in sheep. Multiple serotypes of bluetongue virus (BTV) are enzootic in northern and eastern Australia. However, there has been no evidence to date of naturally occurring bluetongue disease and it has been suggested that this is likely due to the low virulence of Australian serotypes, the low transmission efficiency of the major vector (*Culicoides brevitarsis*) which breeds in cattle dung, and the relatively low numbers of sheep in the bluetongue enzootic zone. The distribution of BTV and its vectors in northern Australia are mapped annually by the National Arbovirus Monitoring Program (NAMP) in support of the livestock export trade and to provide early warning of exotic incursions or changes in distribution of viruses and vectors.

1.2 Bluetongue virus in Europe

Although this has been assumed to be a relatively stable situation, events in Europe in recent years have raised concerns. Historically, Europe was considered largely bluetongue-free, with rare incursions only into the Iberian Peninsula and the Greek Islands. However, since 1996, multiple bluetongue serotypes have invaded from the Middle-East and Africa and become endemic across much of southern Europe. This has been accompanied by an expansion in the distribution of the major vector *Culicoides imicola* that has been attributed in part to global warming. Somewhat more alarming was the unexpected emergence in 2006 of BTV serotype 8 in northern Europe. Although the source of this virus is unclear, and it was expected to be rapidly extinguished, BTV-8 adapted to local Palaearctic *Culicoides* species, successfully over-wintered, and became endemic for several years across much of northern Europe from the UK and France to Sweden and Poland. The establishment of bluetongue in northern Europe coincided with the hottest summer on record.

1.3 Trade impact and gaps in knowledge

These events suggest that the balance of factors that have allowed Australia to remain free of bluetongue disease may be a fragile one that 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 new vectors with a more southerly distribution.
The export trade in live animals is facilitated by the National Arbovirus Monitoring Program (NAMP) which monitors and maps the distribution of arthropod-borne viruses (including BTV) and their vectors in Australia. 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. Bluetongue can cause catastrophic losses in naïve sheep and goat populations. Mortality rates in susceptible flocks can reach 30 -100%. The duration of any disruption to trade cannot be predicted as it would be dependent upon the duration of any disease outbreak and the response of trading partners to the outbreak and control activities. $5.6 billion of meat exports (cattle, sheep & goat) would be at risk per annum plus $1 billion in live animal exports.

There are critical gaps in our understanding of the genetics, biology, ecology and distribution of bluetongue viruses and *Culicoides* in Australia, the genetic determinants of virulence and vector competence and the potential consequences of global warming. These knowledge gaps limit our ability to assess these risks and develop effective measures to prevent or limit the potentially severe impacts on livestock production and trade. This project addresses one of the most critical of knowledge gaps – the identification, characterisation and assessment of risks associated with potential *Culicoides* vectors of bluetongue virus in southern Australia.

2 Projective objectives

The project was designed to allow a preliminary assessment of the likelihood that a European-type scenario could occur in Australia, resulting in the establishment of bluetongue virus in southern sheep and cattle populations. This would provide a basis for improved surveillance and more effective measures to prevent or limit the potential impacts on production and trade. It would also provide the basis for an assessment of the need for further research to better define risks in southern Australia and potential impacts on climate change.

2.1 Objectives

2.1.1 Survey of *Culicoides* species in Victoria, particularly those that occur in grazing country and are associated with cattle and sheep

2.1.2 Determine the host feeding preference of potential vectors

2.1.3 Determine the competence of potential vector species for transmission of Australian BTV serotypes

2.1.4 Assess the risks of BTV transmission in southern Australia

2.2 Outputs

2.2.1 By May 2013

A preliminary assessment of primary *Culicoides* species that associate with sheep and cattle in five different regions of Victoria

2.2.2 By June 2014

1. A species-specific molecular diagnostic tool for southern *Culicoides* species is available
2. A description of the seasonal dynamics of the primary *Culicoides* species in Victoria
3. Quantitative data on feeding preferences of the primary candidate species occurring in the southern Australia
4. Preliminary data on vector competence of the primary vector candidate species

2.2.3 By June 2015

1. Quantitative data on vector competence of the primary candidate species occurring in the southern Australia
2. A relative risk analysis for transmission of BTV in sheep and cattle in Victoria with respect to the host feeding preferences and vector capacity of potential vectors.

2.3 Outcomes

The project leads to:
- A better understanding of the BTV transmission potential of the *Culicoides* species present in the southern parts of Australia.
- A recognition of the risk, if present, by the stakeholders.
- A public policy and a definition of response facing the BTV transmission risk in the southern parts of Australia.

3 Methodology

The project was organised in three elements:

i. identification of potential vectors by sampling of *Culicoides* species in the field, particularly those in close association with cattle and/or sheep;
ii. determination of host feeding behaviour of these species by trapping and host preference studies in the field and molecular analysis of blood meals;
iii. determination of the vector competence of these species by laboratory infection challenges.

3.1 Sampling of *Culicoides* species in the field

3.1.1 Sampling zones

A number of sites were sampled to obtain a representative picture of *Culicoides* species associated with sheep and cattle in Victoria. *Culicoides* in close association with sheep and cattle were screened initially but other species from reserves and parks were included in the distribution analysis.
We focused our sampling on regions with mixed cattle and sheep production: Gippsland, Goulburn Valley and the Central Highlands. Cross-transversal repeated samplings were performed with help from farmers. One longitudinal site in Benalla (Goulburn Valley) with a relatively easy (3 hours) access from AAHL was studied more intensively.

3.1.2 Traps

Several types of trap were used: interception trap (Malaise, SLAM), light traps (CDC light trap with green LEDS (1) and Onderstepoort UV trap) and sticky traps.

3.2 Identification of *Culicoides* specimens

3.2.1 Morphological identification

Morphological identification was used. The Pictorial Atlas of Australasian *Culicoides* Wings (2) has been widely used as reference. An electronic key was built for initial training. Links with Glenn Bellis (AQIS – Darwin) who has recognised expertise in these methods were

accessed for quality checking. The diversity of *Culicoides* is estimated by the Shannon index, estimating the number and equality of proportions of taxa in a given environment.

3.2.2 Age grading

Under binocular magnification, specimens were graded according to the abdomen status (blood fed, pigmented, gravid or clear). The 'clear' specimens were classified as unparous midges. Only populations providing at least 25 specimens were graded.

3.2.3 Molecular identification

Molecular diagnostic markers (mitochondrial and nuclear) were used for accurate species/subspecies identification of potential vectors. Collaboration with David Gopurenko (DEPI - NSW) was very useful.

DNA Extraction

Individual *Culicoides* were removed from ethanol and allowed to air dry before extraction(3). DNA was extracted using two methods:

- The MagMaxTM-96 DNA Multi-Sample Kit (Applied Biosystems, Austin, TX) on a KingFisherTM Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA). Isolation of genomic DNA from mouse tail protocol in a 96-well plate format was utilized with the following amendments. *Culicoides* were individually incubated in 100 µl of PK buffer and 10 mg/ml of Proteinase K per sample in a 2 ml Sarstedt tube. Samples were homogenized by bead beating with a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA) at 6.0 m/s for 20 s with approximately 10 x 1.0 mm zirconium/silica beads (BioSpec Products, Bartlesville, OK) before overnight incubation at 56°C. Elution was performed using 40 µl of elution buffer 1 and 40 µl of elution buffer 2. To assess possible contamination between wells during the KingFisherTM extraction procedure, every eleventh well in the MagMaxTM Deep Well 96-well plate was left without a *Culicoides* sample, but was still included in the downstream screening.
- Alternatively, DNEasy Blood & Tissue kit (Qiagen) with small modifications of the manufacturer's protocol: *Culicoides* were individually incubated overnight without homogenisation at 56°C in 200 µl of lyses buffer with Proteinase K in a 2 ml Sarstedt tube; after incubation, the midge was removed and stored aside in alcohol 70% for morphology voucher at -20°C; finally the last elution was done twice with a low volume (50 µl) for a better DNA yield.

Polymerase Chain Reaction (PCR) screening

Primers (GeneWorks, SA, Australia) for the amplification of the COI (cytochrome oxidase subunit 1) were obtained from Glen Bellis (4). Reaction mix setup was performed using 1 unit of Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and 1 µl of template. Two negative controls were included for all PCR screens to ensure no contamination occurred. PCR amplification was performed with an initial denaturation at 94°C for 2 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. A single positive control was included for each PCR; DNA from *Culicoides victoriae* was used. Amplified products were

separated on a 1% agarose gel containing 0.1% SYBR® Safe DNA Gel Stain (Invitrogen, USA) and visualized with a G:BOX Syngene blue light visualization instrument.

Sequencing

COI amplicons were sequenced for *Culicoides* species confirmation. DNA was purified from positive amplicons with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Fluorometric quantification of purified DNA was performed using a dsDNA HS Assay on a Qubit® (Invitrogen, USA). Sequencing was performed using the BigDye® Terminator v3.1 kit (Life Technologies) according to the manufacturer's protocol and sequence was generated via capillary Sanger sequencing on a 3500 Genetic Analyser (Applied Biosystems).

Restriction Fragment Length Polymorphism (RFLP)-PCR

Consensus sequences were obtained for the 5 most commonly collected *C. victoriae* related taxa species using the outlined COI primer set, amplifying a 692 bp region of the COI gene. Consensus sequences were obtained using the Clustal W algorithm in Geneious version 8.0.5 (Biomatters Ltd) and examined for recognition sites of a combination of 52 different restrictions enzymes. Restriction enzyme TaqαI (New England BioLabs® Inc.) was selected with TaqαI providing sufficient differentiation between the *C. victoriae* species as depicted in Figure 1. Amplified product from the COI PCR was used directly for the RFLP analyses, without performing PCR purification. Digestions were performed in a total volume of 15 µl using 8 µl of amplified product, 1.5 µl of 10 X CutSmart® Buffer (New England BioLabs® Inc.), 1.5 µl of 10 X BSA (New England BioLabs® Inc.), 1.5 µl of ultrapure water. TaqαI (New England BioLabs® Inc.) was diluted fresh for each use to 0.02 U/µl in ultra pure water with 2.5 µl being added to each digestion, before incubated at 65°C for 1 h. Restricted fragments were analysed by electrophoresis on a 2% agarose gel in 1 x TAE buffer containing 0.1% SYBR® Safe DNA Gel Stain (Invitrogen, USA) and visualized with a G:BOX Syngene blue light visualization instrument. Both the digested and undigested products were run in parallel to confirm the amplification and digestion were successful.

3.2.4 Ecological analysis

A matrix of *Culicoides* species x sites was built with the data from Green LED CDC light traps catches. General indices of diversity (species richness, Shannon index) were arranged by site. Clustering of sites according to species abundances was analysed by correspondence analysis followed by calculation of Euclidean distance from dissimiliraty coefficient by coefficient of Gower and Legendre (5)in a quantitative variable matrix and clustering with average link (UPGMA) hierarchy algorithm in ADE4 sofware (6).

3.3 Host-feeding preferences

Three approaches were used to obtain data on host feeding preferences of potential vector species:

i. Trials of aspiration of midges around cattle and sheep.
ii. Urine-baited traps were used modified for use with *Culicoides* to offer a choice of host odours. This approach will provide data on the upstream host-seeking phase of the feeding behaviour which is more related to the genetic background.
iii. Blood meals from blood-fed female *Culicoides* were analysed by molecular techniques (PCR).

3.3.1 Aspiration around animals

Despite agreement of the Animal Ethics Committee, this method was quickly stopped as animal owners did not wish to have animals disturbed excessively (immobilization, mechanical aspiration noise…).

3.3.2 Baited traps

To provide data on *Culicoides* feeding behaviour, urine-baited traps were installed and operated in a Latin square rotation scheme offering different urines, or water as a control to test the attractiveness of chemicals odours of mammalian origin(7). CDC light traps with green LEDs were baited with approximately 100 ml of aged urine (10-21 days) from either cattle (4 animals) or sheep (~10 animals) displayed close to the entry of the traps. Traps were 25-50 m distant and baits were rotated each night. Six sites were sampled:

- The initial site was at AAHL (swamp area) in Geelong during 6 nights where the conditions of sampling were set up.
- Benalla (Goulburn Valley, sheep farm) – at 2 sites 300 m distant during 3 nights.
- Glenaladale (Gippsland, sheep farm and forest bush), during 3 nights
- Stratford upon Avon (Gippsland, sheep farm) during 3 nights.
- Later in the season, experiments were conducted in the Northern Territory (NT) to check the method for trapping of known *Culicoides* vectors (*C. brevitarsis)* breeding in cattle dung at two sites: Beatrice Hill (buffaloes) and Darwin (horse farm) during three consecutive nights at each site.

3.3.3 Blood meal molecular analysis

DNA from blood fed-females was extracted by using the DNEasy Blood & Tissue kit (Qiagen) with small modifications of the manufacturer's protocol (see 3.2.3). A PCR targeting the *cytochrome b* gene of vertebrate origin (primers Cyt b L14841F and H15149R) was used after Peterson *et al.(8)*. The obtained sequences were identified using BLAST against the NCBI database. As far as possible, morphological vouchers were stored for further confirmation of the identification. DNAs from either bovine blood or blood-fed *Culicoides imicola* collected in Madagascar near zebus were tested and used as controls for bovine origin. DNAs extracted from sheep meat were used as the ovine positive control.

3.4 Vector competence

The vector competence of the primary potential vector species was assessed in laboratory studies performed at AAHL. This was accomplished with the help of Gert Venter, from Onderstepoort Veterinary Institute in South Africa, who visited AAHL for two weeks from mid-February 2014.

3.4.1 Midge collections

Onderstepoort UV traps were set up in Benalla for 33 trap-nights. Traps were set for the whole night close to resting animals (sheep, horses) in paddocks. Insects were collected alive, self-sorted (alive) in the early morning through fine mesh into small cardboard containers. They were provided with 10% sugar solution and placed in iceboxes with cold packs, then transported by car to AAHL. Once in the laboratory, they were allowed to rest for

2 to 6 days for adaptation (23^{o}C – relative humidity was increased with wet towels) before experimental infections. Sugar was provided *ad libitum* but ceased 24 h before infective meals.

3.4.2 Virus infection

After several membrane trials (Parafilm, collagen membrane, and chicken skins), midges were offered an infective blood meal through 1 day-old chicken membrane feeding using a Hemotek device. Bluetongue virus serotype 1 (strain CS 156, our recent works show it as representative of segments of serotype 1 and 21, if not considering the segment responsible for genotypes) supernatant culture was diluted in heparinised cattle blood (naïve for BTV) to a final viral titre between 3 x 10^5 and 1 x 10^6 $TCID_{50}$/ml. Midges were first set in environmental cabinets at 15^{o}C, 50% relative humidity and darkness in small plastic containers in direct contact with chicken skin (unfeathered). Midges were allowed to feed for 30-60 min. The midges were then anesthetised with CO_2 and sorted on a chill table. Freshly blood-fed *Culicoides* were reserved in cardboard cups, with 10% sugar and maintained at 23^{o}C in darkness for 12-13 days of extrinsic incubation. Unfed *Culicoides* were returned to containers for re-testing on the following day. At the end of the extrinsic incubation period, live midges were anesthetised with CO_2 and sorted on the chill table, identified at the species level and stored at -80^{o}C in individual tubes. At same time, for each container, the first layer of sugar-impregnated filter paper which was in contact with midges' saliva was stored at -80^{o}C.
Positive and negative controls have been defined by *Culex annulirostris* mosquitoes infected with the same BTV-1 strain either orally (negative controls) or by intra-thoracic microinjection (positive controls).

3.4.3 Virus assessment

Samples were homogenized in 200 µl of MEM for insect cell culture by bead beating with a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA) at 6.0 m/s for 20 s with approximately 10 x 1.0 mm zirconium/silica beads (BioSpec Products, Bartlesville, OK). An aliquot of 100 µl was stored in -80^{o}C for virus isolation (C6/36 cells). The remaining 100 µl was mixed with Magmax nucleic acid extraction buffer for both DNA and RNA extractions. Positive controls have been defined by serial dilution ($TCID_{50}$: 10^{-4} to 10^{-1}) of virus mixed with individual unfed midges. BTV-1 RNA presence and quantification was assessed by using a BTV real-time TaqMan PCR assay targeting the segment 10 that is routinely run at AAHL (9). The assays are run in duplicate on an Applied Biosystems model 7500 Fast or 7900HT. A separate TaqMan assay targeting 18S ribosomal RNA is conducted in parallel with the BTV-specific TaqMan assay as an internal control assay. This allowed validation of the RNA extraction procedure, determination of the integrity of the RNA sample and confirmed the absence of significant levels of PCR inhibitors. 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-ARGCTGCATTCGCATCGTACGC-TAMRA-3'). CT values higher than 40 were considered negative. Duplicates were required to yield the same results for a firm conclusion. Specimens giving positive and negative results were treated as inconclusive. Results were expressed as prevalence of positive specimens and mean CT values. They were compared

with starting CT values of freshly fed insects. Given the results of this molecular assessment, sheep-vector-sheep transmission experiments were not conducted.

3.5 Risk analysis

3.5.1 Elements

We considered vector capacity as the best measure of transmission risk (10). This includes measures of parous rate, the estimation of host range, the vector competence assessed by published reports and our experimental data, and the comparisons of population abundance (total numbers and relative % ages).

3.5.2 *Culicoides* species

The species most abundantly found in Victorian farm environments were chosen for the risk assessment (*C. austropalpalis, C. marksi,* and *C. victoriae*). *C. brevitarsis* and *C. actoni* were chosen as positive controls.

4 Results

4.1 *Culicoides* sampled in Victoria

4.1.1 Traps yields

All differences between traps were significant, except between the Green LED CDC light traps (GLED) and Onderstepoort UV traps (UVSA). UVSA = GLED > sticky traps > interception traps. The Green Led CDC light traps were more reliable during the study: they employ batteries and so are independent of an external electric power supply. They are almost identical to those used in the National Arbovirus Monitoring Program. However, due to their slightly higher yield, the Onderstepoort UV traps were used for collection of large numbers of midges for experimental infections.

Table 1. Comparison of the average mean yield rate between traps, expressed as the number of culicoides per trap per night.

Trap Type	N	Mean	SD
GLED	191	120.8	333.0
Intercept	53	0.47	1.47
Sticky	197	9.0	26.6
UVSA	16	242.1	568.1

4.1.2 Species present

A total of 24 taxa were collected. Of these, four (*C. bunrooensis, C. ornatus gp, C. waringi* and *C. williwilli*) were newly known for Victoria but were already known in neighbouring states (NSW or South Australia). Five new taxa were closely related to known species but, after analysis of molecular data (see under), were considered to be new species : *C. molestus* gp (foreseen name: *C. balhorni*, work in progress with G Bellis), *C. bundyensis-like* (work in progress G Bellis), and *C. victoriae-like* (three new taxa – work in progress – one is mentioned later in the analysis as *C. victoriae-like 2*). Four taxa mentioned in the Pictorial

Atlas of Australasian *Culicoides* Wings (2) were not found during our collections (*C. mcmillani, C. molestus gp3, C. victoriae gp15* and *C. williwilli gp18*).

4.1.3 Abundances

There were large differences in the total number of caught culicoides between sites, even at small distance scale. However, the south-west region of Victoria showed low values.

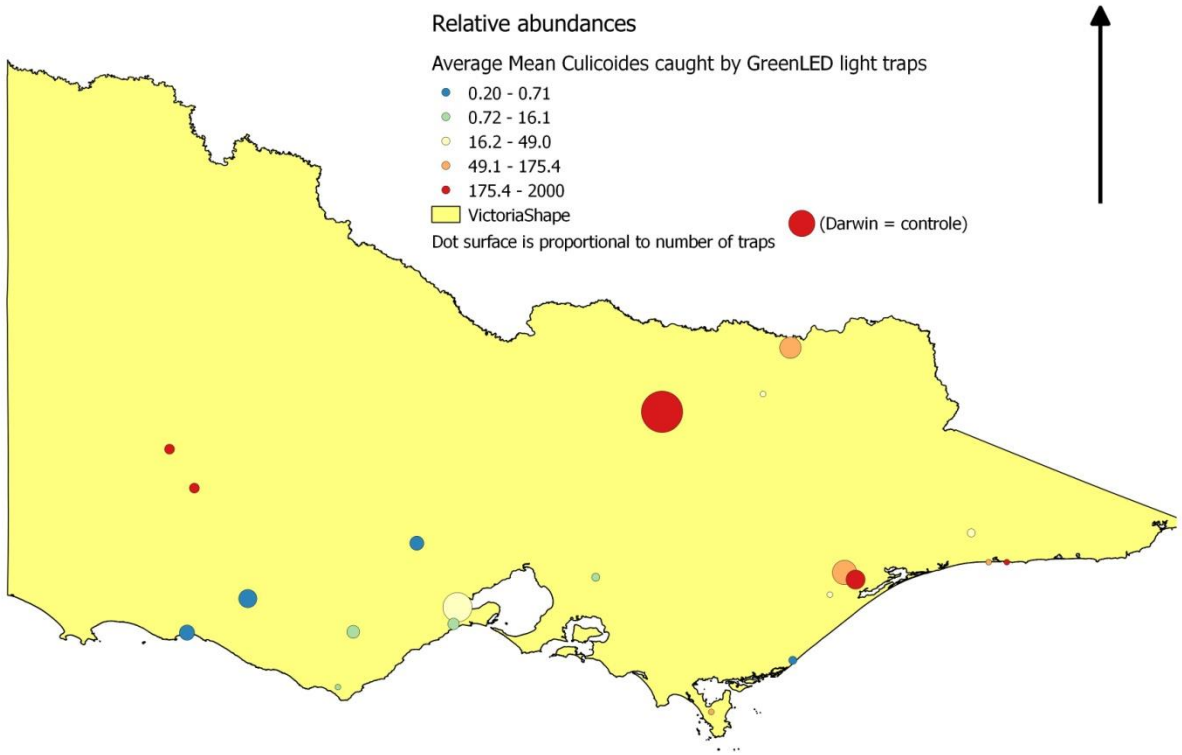

Figure 1. Map of relative abundances of *Culicoides* (all species) in Victoria. Culicoides were caught by using Green LED CDC light traps. The dot **colour** is proportional to the **average count** of culicoides per trap (red high - blue low). The dot **area** is proportional to the **sampling effort** (smaller = 1 trap-night). Note that the dot for Darwin placed near legend as control is geographically incorrect.

The most abundant species was *C. austropalpalis*. The victoriae group as a whole (C. *bundyensis-like, C. victoriae-like, C. waringi, C. multimaculatus*) was abundant and diverse in Victoria. Even if appearing abundant in the chart (Fig. 2), *C. molestus gp* (*C. balhorni* - in green) was extremely restricted in distribution (rivermouth of the Snowy river). The *Marksomyia* subgenus includes two main species which were relatively abundant: *C. marksi and C. dycei*.

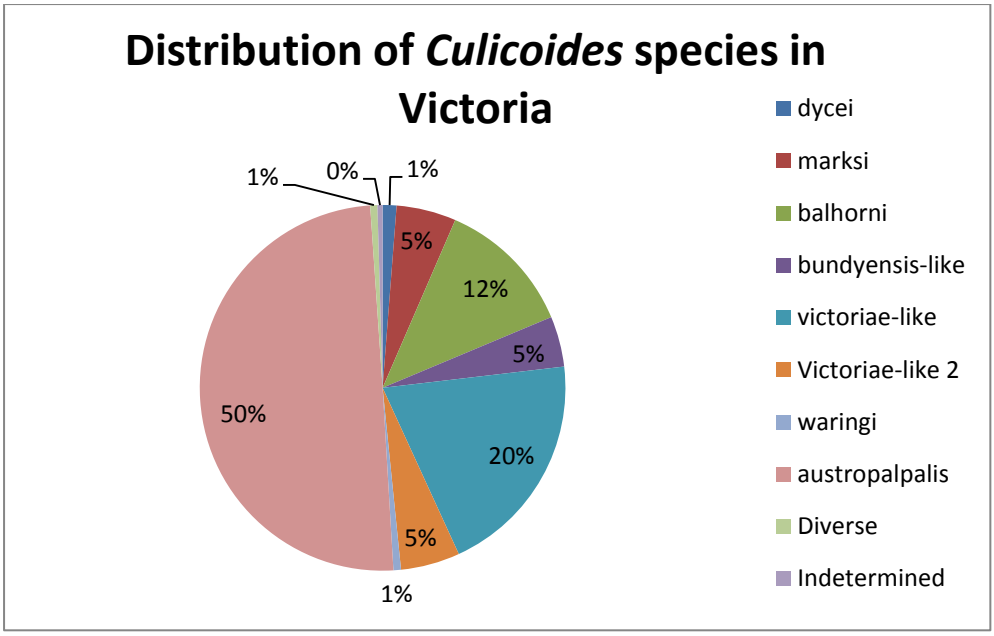

Figure 2. Relative distribution of *Culicoides* species in Victoria. Species collected in low numbers (<100 specimens) were grouped together under 'Diverse'.

4.1.4 Age grading

There were large differences in parous rates of different species. Several species presented very high parous rates (>80%): *C. balhorni, C victoriae-like2,* and *C. waringi*. Moreover, we could not find any bloodfed or pigmented specimens of these species. So, for these species, the high parous rate was exclusively caused by gravid females. *C. multimaculatus* showed the same pattern but few pigmented specimens were found. The most abundant species (*C. victoriae* group and *Marksomyia*) showed high parous rates, higher than 65%, and comparable to the known vector *C. brevitarsis* in NT (67%). Globally the parous rates were higher in Victoria than in NT. The most abundant species, *C. austropalpalis*, had a parous rate of lower than 50% in both Victoria and NT.

4.1.5 Ecology

The site in Victoria with richest species diversity was Benalla, with 13 species recognised, followed by Wodonga (9 species), the Emu farm, near Stratford on Avon (8 species) and Glenaladale (7 species). The higher Shannon indices were found in Glenaladale, Grampians and Wodonga. The sites with highest values are situated on the slopes of Victorian mountain ranges (the Grampians, Glenaladale, Wodonga). However, close sites within the same region (Gippsland, around the Grampians) could have lower values. Several coastal sites combined low Shannon index and low richness of diversity with a relatively high abundance. The NT site (Darwin region) combined the highest Shannon index and richness of speciied diversity. It highlighted the well-known latitudinal effect for diversity (high in equatorial zones and low near poles).

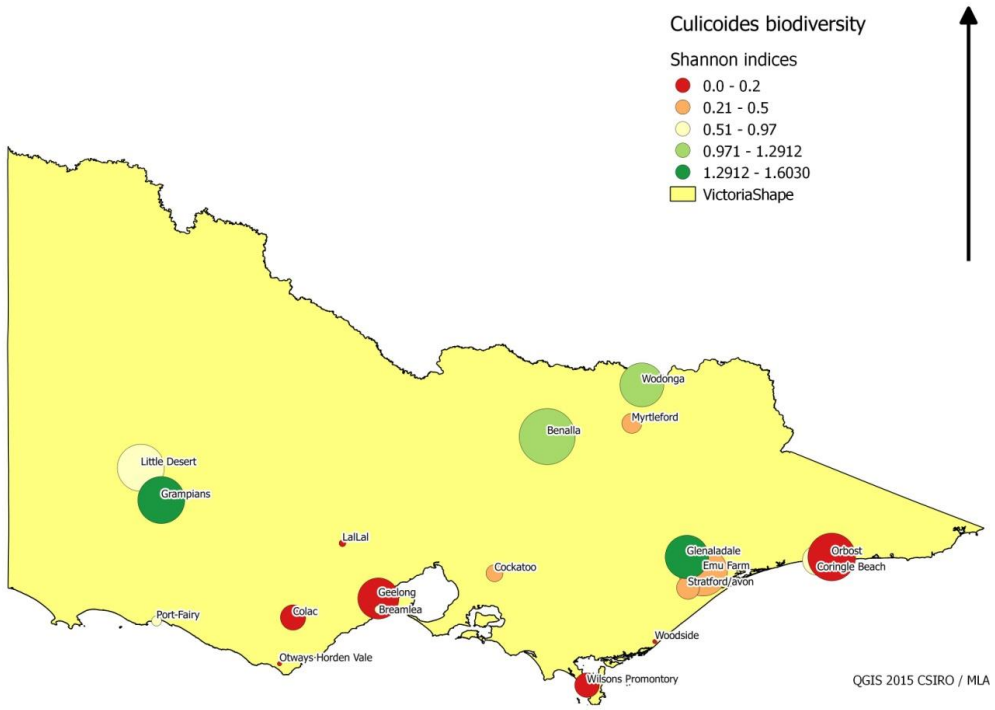

Figure 4. Map of Shannon indices by site. *Culicoides* were caught by Green LED CDC light traps. The dot colour is proportional to the Shannon index site (red low - green high). The dot area is proportional to the total count of culicoides per site.

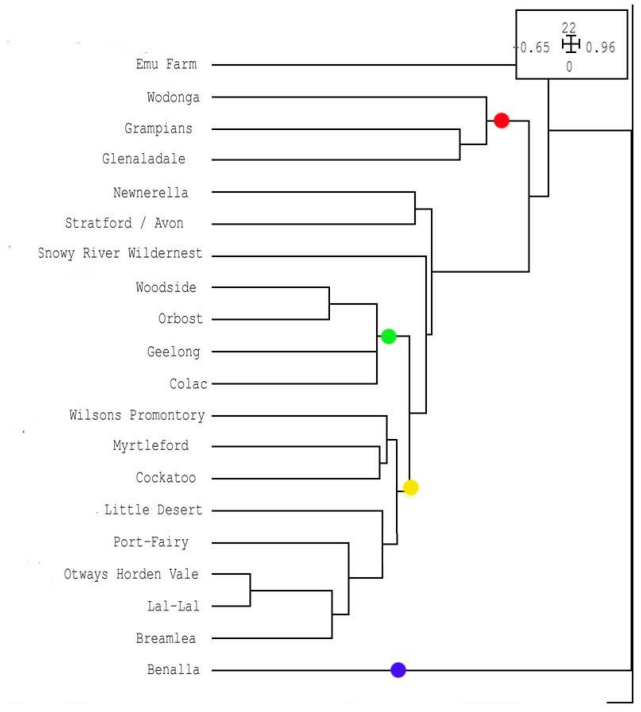

Figure 5. Cluster analysis of sites according to *Culicoides* abundances by species.

Analysis of the patterns of distribution of *Culicoides* species by clustering after computation of dissimilarity of sites allowed the classification of sites in a tree (Fig.5). The site at Benalla, where the *Culicoides* fauna was the most abundant and relatively diverse was well apart

(blue dot). The most diverse sites clustered in one branch (red dot). The green-doted branch combines coastal sites from East Gippsland with Geelong and Colac. This was due to species (*C. victoriae-like2, C. waringi* and C. *bahlorni in press*) from estuarine and saline environments which could be relatively abundant but do not share their environment with other species. The yellow-doted branch groups sites where *Culicoides* are neither abundant nor diverse.

4.2 Molecular identification

A large morphological diversity was seen to occur amongst the *C. victoriae-like* insects collected, with species being grouped to one of five morphological variants. A RFLP-PCR method was developed and implemented as a better species diagnostic.

RFLP of *C. victoriae* group

Based on consensus sequence data of the five *C. victoriae* morphological variants, restriction enzyme Taq$^{\alpha}$I on PCR products was determined to provide sufficient species discrimination. All *C. victoriae* samples tested produced RFLP profiles readily distinguishable into one of the five species groups. Species typing capability of the RFLP-PCR was confirmed by sequencing *C. victoriae* samples from multiple locations, where possible. Sequences were successfully obtained for *C. victoriae* A (n=25), B (n=20), C (n=17), D (victoriae-like2, n=13) and E ("true" C. victoriae caught in the type locality, Cockatoo, n=3). A Phylogenetic tree was constructed for the 5 *C. victoriae* group, in which each group separated into independent branches (Fig. 6).

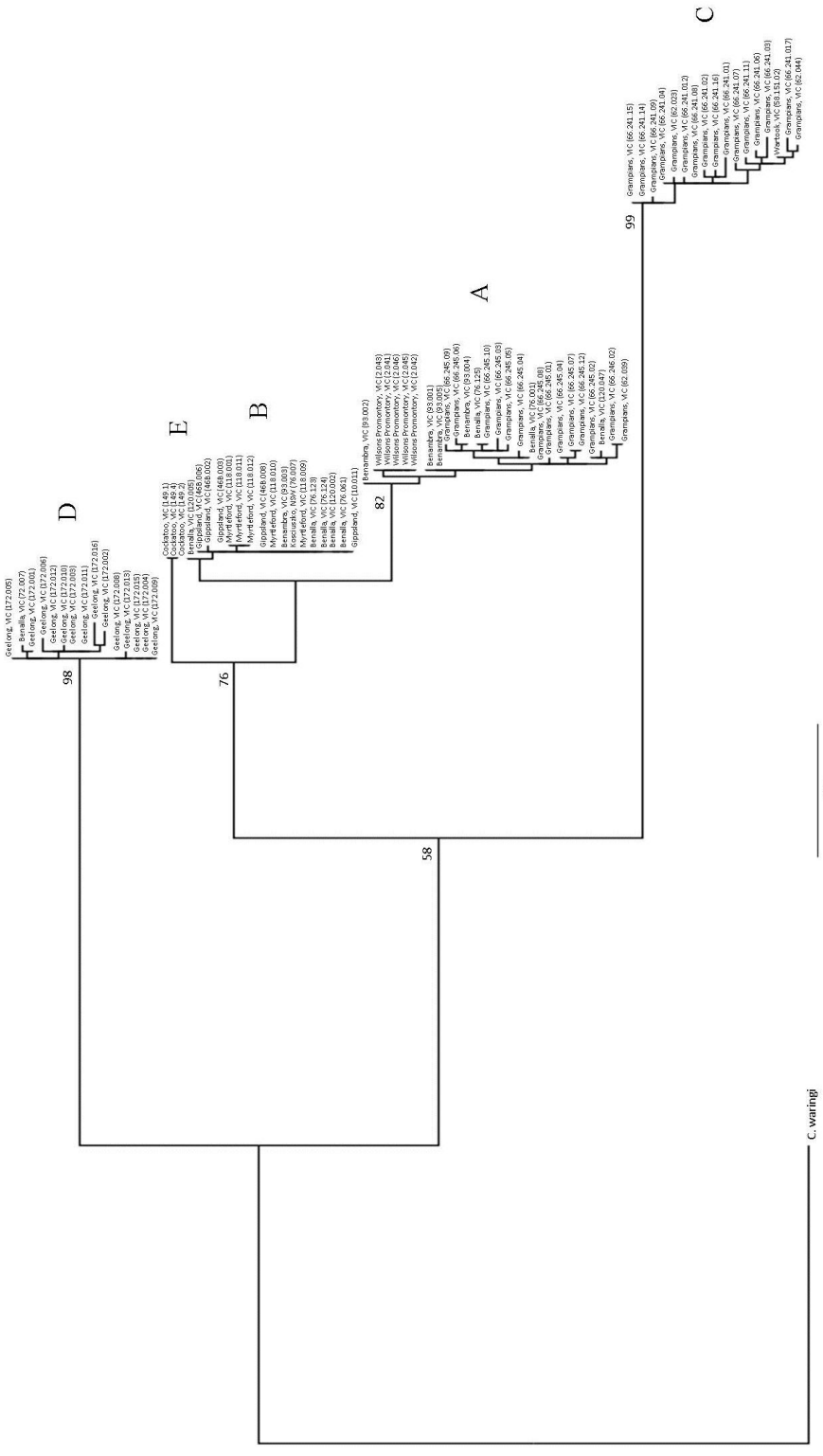

Figure 6. Maximum-likelihood phylogenentic tree of the five *C. victoriae* groups. Based on a 378 bp region of the COI gene. Nucleotide sequences were aligned using the Clustal W algorithm employing the General Time-Reversible (GTR) model, based on JModelTest2 analysis with 1,000 bootstrap replicates. Bootstrap proportions (BSP ≥70%) are indicated beside nodes. *C. waringi* was used to root the tree; all sequences were generated by this study.

The *C. victoriae* RFLP-PCR gave a 92% success rate in correctly identifying a *C. victoriae* individual to one of the five groups, based on sequence confirmation. The 8% of samples which failed to be correctly identified were due to 6 *C. victoriae* individuals from Wilsons Promontory, Victoria, all presenting a single nucleotide change in the Taq$^{\alpha}$I restriction site, resulting in the incorrect RFLP banding pattern. The lowest sequence divergence was seen between *C. victoriae* species A and B, having an average sequence similarity of 95.11%, with all other species groups having a higher level of divergence.

4.3 Host preferences

4.3.1 Urine-baited traps

A total of 10,834 female and male *Culicoides* were trapped during these experiments: 41.2% by using water, 28.7% by using sheep urine and 30.1% by using cattle urine. These proportions were different between the Northern Territory and Victoria, with a relatively balanced pattern in NT, respectively 35.0%, 34.5% and 30.4% for water, cattle and sheep urine, respectively; and a significantly different (chi-square $p<0.0001$) pattern in Victoria: 48.3%, 25.0% and 26.7%, respectively. When aged (parous) and young females were differentiated, the difference was still significant between NT and Victoria and, within each of these states, between cattle urine and water. This means that a greater proportion of aged females were caught with cattle urine bait than water, and that this difference was higher in Victoria than in NT. Expressed differently, young females were more reluctant to go to cattle urine than water in Victoria than in NT. Considering sheep urine, parous female *Culicoides* tended to be significantly more attracted (chi-square 2-tailed $p<0.015$) than younger compared to water, but only in Victoria, and not in NT.

Table 2. Statistical significance of the difference in proportions between parous and unparous females according to trap baiting (chi-square two tailed) and state (Breslow-Day test between chi-square strata).

		NT		Victoria		Difference between states (Breslow Day test)	
	Chi-square 2-tailed p values	Cattle/ water	Sheep / water	Cattle/ water	Sheep / water	Cattle/ water	Sheep / water
All species	aged/young	**0.046**	0.48	**<0.0001**	**0.0135**	**<0.0001**	**0.0253**
C. marksi	aged/young	0.0952	0.1207	**0.0007**	0.9181	**0.04314**	0.191
C brevitarsis	aged/young	0.2505	0.06006	N/A	N/A	N/A	N/A
C. victoriae	aged/young	N/A	N/A	**0.006**	**0.00297**	N/A	N/A
C. austropalpalis	aged/young	**0.0086**	0.6385	**<0.0001**	0.3	0.05951	0.871

Considering differences in the abundant species, the difference observed between states for the cattle urine bait was significant for *C. marksi* only. However, all the three species tested from Victoria (*C. marksi, C. austropalpalis* and *C. victoriae)* showed a significant trend for young female to be more repulsed by cattle urine than aged females. This is also true for *C. marksi* in NT. Concerning sheep urine, only *C. austropalpalis* in Victoria presented the same

behaviour for young females. In NT, for all the tested species, the young did not present any difference from older females, although *C. brevitarsis* was close to statistical significance.

Concerning the differences according to sex, the males tended to be strongly repulsed by cattle urine. This relationship did not exist for sheep urine in the NT, all species together or each species separately. By species, *C. austropalpalis* males in Victoria were significantly repulsed by cattle and sheep urine. *C. marksi* presented a similar pattern in Victoria but not significantly. However, this species is strongly influenced by cattle urine in the NT.

Table 3. Statistical significance of difference of proportions between males and females according to trap baiting (chi-square two tailed) and state (Breslow-Day test between chi-square strata).

		NT		Victoria		Difference between states (Breslow Day test)	
	Chi-square 2-tailed p values	Cattle/ water	Sheep / water	Cattle/ water	Sheep / water	Cattle/ water	Sheep / water
All species	sex-ratio	**<0.0001**	0.4959	**0.0001**	**<0.0001**	0.6504	**0.000254**
C. marksi	sex-ratio	**0.0079**	0.4575	0.0636	0.07836	0.5891	0.265
C brevitarsis	sex-ratio	0.1567	0.4818	N/A	N/A	N/A	N/A
C. victoriae	sex-ratio	N/A	N/A	0.1169	0.3485	N/A	N/A
C. austropalpalis	sex-ratio	0.2306	0.1488	**0.0001**	**<0.0001**	0.1072	**8.39E-05**

4.3.2 Blood meal molecular analysis

The molecular analysis of blood-meals provides a very precise diagnostic of host species, based on the cytochorme b databank (life barcoding). *Culicoides* as taxonomiccally grouped tended to feed on related hosts (victoriae group species on marsupials, *C. austropalpalis* on birds). *Marksomyia* subgenus species showed a range of prey mostly related to large placental mammals of exotic origin, including livestock - historically - and humans – prehistorically -, as do the *Avaritia* subgenus species, present in NT (*C. brevitarsis, C. actoni* and *C. imicola* from Africa).

Table 4. List of blood meal origin by species. In red, blood meal of livestock origin; in pink from humans; in green from marsupials, in yellow from birds. Most of birds are endemic to Australia or the region.The *Culicoides* species are classified by taxonomic groups; in blue, species which are found in Victoria.

	Cow	Buffalo	Goat	Homo sapiens	Eastern grey kangaroo	Swamp wallaby	Wombat	Brown Thornbill	Egret	Magpie	Red-browed Finch	White-breasted woodswallow	Willy wagtail	Yellow-plumed Honeyeater
C. actoni	1	2		1										
C. brevitarsis		2		5										
C. imicola	5			1										
C. histrio									1					
C. oxystoma		1												
C. peregrinus				1										
C. bundyensis						1	1							
C. victoriae				1	3	2								
C. dycei				4										
C. marksi	1	8	1	3								1		
C. ornatus gp#6		1												
C. austropalpalis				1				1		11	3		1	1

4.4 Vector competence

Here are presented the results of the experimental infections perfomed in the laboratory on *Culicoides* collected from the field in Victoria. Among *the Culicoides tested, 94.5 % were C. austropalpalis, 1.7% C. marksi, 1.2% C. victoriae, 0.7% C. bundyensis*, 0.7% *C. ornatus* gp and 1.2% were not determined.

4.4.1 Blood-feeding and survival rate

The rate of blood-feeding for *Culicoides* was 49.8% (n=3293). For comparison, the rate for mosquitoes was 90.6% (n=53). The percentage survival for *Culicoides* at day 13 was 25.2% (n=1641), and 58.3% for mosquitoes.

4.4.2 Quantitative PCR results

The average mean of Ct values for freshly bloodfed *Culicoides* was 30.9 (n = 12, SD = 1.98) and 26.12 for mosquitoes, n = 2, SD = 0.99). The difference was significant (p = 0.007). The average mean of Ct values for microinjected mosquitoes was 24.2 (n = 2, SD = 0.16).

The prevalence of positive specimens (both CT<40) was 7.4% (31/416) for culicoides, 0% (0/14) for bloodfed mosquitoes and 100% (9/9) for microinjected mosquitoes.

Table 5. Prevalence of positive, negative and inconclusive Ct values for BTV-1

	Positive (%)	Negative (%)	Inconclusive (%)
Culicoides BF	31 (7.4)	338 (81.3)	47 (11.3)
Mosquitoes BF	0 (0)	12 (85.7)	2 (14.3)
Mosquitoes microinjected	9 (100)	0 (0)	0 (0)

Of 31 positive specimens, 94% (30) were *C. austropalpalis*, and 6% were respectively *C. marksi* (1) and undetermined (1). The positive Ct values for these two species were not different from those for *C. austropalpalis*. All the inconclusive samples were *C. austropalpalis*.

Table 6. Mean and standard deviation for positive and inconclusive Ct values

	Positive		Inconclusive	
	Mean (n)	SD	Mean (n)	SD
Culicoides BF	35.1 (31)	1.42	40.54 (47)	0.527
Mosquitoes BF	0 (0)		40.59 (2)	0.191
Mosquitoes microinjected	20.59 (9)	2.90		

The positive Ct values after the extrinsic incubation was significantly ($p<0.0001$) higher than after a fresh blood meal. The difference for microinjected mosquitoes was not significant ($p=0.08$)

4.5 Risk analysis

4.5.1 Parameters underlining vector capacity

The most useful historical references in the context of blood meal origin in *Culicoides* are Muller et al (11) and Muller & Murray (12). Our new method for baiting traps with urine has not been used before in Australia (or elsewhere) and so provides original data. Previous data on experimental infections come from Standfast et al (13). Other work has been published since this date (14); (15); (16). However, they do not bring new conclusions and so for clarity they have not been included in Table 7. For better comparsion between species and regions, we have preferred to use only our trapping data for estimations of abundance. Large differences in abundances were recorded between and within zones. However, general trends could be identified: *C. marksi* was more prevalent and abundant in the tropical North than in temperate southern zones of Australia; *C. austropalpalis*, *C. victoriae* predominated in the South but were rarer in the North. Like abundances, the parous rate

data were derived from our work and could be compared between species and between states.

4.5.2 Vector status

If we consider each species, some do present problems:

- *C. marksi* shows a clear preference for large placental mammals and a high parous rate in the temperate zones. It could be relatively abundant. However, the published data on experimental infections are convincing and suggest an absence of vector competence for the tested BTV serotypes 1 and 20. The recent revision of the *Marksomyia* subgenus confirms its status of one unique species across Australia.
- *C. austropalpalis* is very abundant and found frequently in the Victorian farm environment. However its parous rate, its taste for birds and our experimental data make it a very poor, if not null, candidate for BTV transmission. Our molecular data confirm it as one species in Victoria. However, unless care is taken, it may be confounded with *C. ornatus* gp.
- *C. victoriae* is analysed as a whole. It can be abundant in Victoria and its parous rate could be high. However, from our results, it mainly feeds on marsupials. However, it has been found to feed upon ox and sheep in New South Wales. The data from Muller (17) do not exclude the possibility of a minor vector status for BTV 1. The most critical point is the presence of a complex of species comprising variants that may vary in key properties that affect vector capacity.
- *C. bundyensis* is analysed as a whole. It is not so abundant as *C. victoriae* but its parous rate is relatively high. It has been found to feed mainly on marsupials but could bite sheep and cattle. Experimental infection data from Standfast in the NT and Kay did not show any vector competence for BTV 1 and 20. However, like *C. victoriae*, *C. bundyensis* is a complex of species.

Table 6. Summary of parameters important for vectorial capacities (data from literature and current work)

	blood meal		baited traps		Experimental infection	Virus isolation	Abundance (our collections)		parous rate	
	referenced	ourselves	sex-ratio	aged			Victoria	NT	Victoria	NT
C. brevitarsis	sheep and ox (Muller 1977 a&b)	bovids and human	no difference	no difference	positive (Standfast 1978)	Yes	0	++ (26%)	N/A	67%
C. actoni	Bovids (Muller a)	bovids and human	female -> cattle	young -> sheep	positive (Standfast 1978)	Yes	0	+ (8%)	N/A	51%
C. marksi	sheep and ox (Muller 1977 a & b)	bovids and human	female -> cattle & sheep	aged -> cattle in Victoria	Negative N=1812 (Standfast 1978)	No	+ (6%)	++ (17%)	82%	52%
C. victoriae	sheep and ox (Muller 1977b)	marsupials	no difference	aged -> cattle & Sheep	Negative N=230+45 (Muller 1993)	No	++ (14 %)	~ 0 (0.1%)	81%	N/A
C. bundyensis	88% marsupials Muller 1977	marsupials	N/A	N/A	Negative N=1265 Standfast 1978	No	+ (6%)	+/-(2%) Standfast 1984	68%	N/A
C. austropalpalis	Birds (Muller 1977a, Kay 1978)	Birds	no difference	aged -> cattle	Negative N=393 (ourselves)	No	+++ (64 %)	++ (24%)	40%	39%

5 Discussion

5.1 Inferences and insights of the data

5.1.1 *Culicoides* in Victoria

The data the project brings are important: previous and current data on *Culicoides* in Victoria rely on the NAMP but only a very limited number of sites (2-5 according to years) are sampled by the program. Similarly, historical data are also limited in their magnitude, are outdated and limited geographically. This study has generated new data by adressing the State of Victoria as a whole with the best possible representative sites:

- New species have been found in Victoria that were previously described in neighbouring states. This may reflect the intensive sampling effort during the project which improved the chance of capture but these specimens were very rare: less than 10 for each species. Climate change could have caused a shift in species distribution. However, it is difficult to exclude accidental migration of specimens from other peripheral zones. No males were found for these rare species which would have suggested proximity of breeding sites and local installation. However we have to consider the exception of *C. waringi* which is found near hypersaline inland lakes. This very particular biotope could have been by-passed by previous sampling.
- Several relatively abundant endemic species (*C. victoriae*, *C. bundyensis*) are indeed complexes of species and molecular data allowed us to take the first steps in deciphering these complexes. Importantly, such complexes have been found among palearctic *Culicoides* species in temperate Europe, with varying vector competence

for BTV. However, unlike the Victorian species complexes, the European species belong to the *Avaritia* subgenus which includes known BTV vectors.

- Several species found in coastal (*C. victoriae*-like 2 or gp D in the Phylogenentic tree shown in Fig. 3), estuarine (*C. balhorni* in prep.) or saline (*C. waringi*) aquatic environments show very high parous rates. These high rates are biased by the absence or rarity of young teneral females with clear abdomens. Also very rare are the bloodfed (which are usually the rarest status of *Culicoides* females in our series and elsewhere) and pigmented females. The systematic triggering of pigmentation after blood meal and egg-laying is discounted by authors as being not constant for some species. We propose that this pattern in female populations corresponds to autogeny. This mechanism is known for some species and means that the egg production, most often only the first batch does not have to rely on blood meal and is trigerred after emergence of the adult from pupae. This mechanism is also observed in mosquitoes, often in temperate climate species with a limited season of optimal growth and offering an initial boost of population dynamics, which could bias the relative abundance (like for *C. balhorni*). The mosquito species are often associated with a poor vector capacity as the first blood meal is post-poned during the life cycle.
- The most abundant species is *C. austropalpalis* and we will discuss its associated risk later. At some sites in Victoria, *Culicoides* abundance was comparable to northern tropical areas. However, these levels corresponded to peaks of abundance which were very limited in time and space, representing hot bursts of *Culicoides* population size (18).The scale of these peaks makes spatial definition of abundance very difficult. The scale effect is major when large differences occur within a limited spatial scale and so extrapolation to the state level is problematic. However, our data have revealed some trends: the diversity of *Culicoides* species is very limited in coastal areas and it could be maximal in zones close to relief (slopes) with coverage of native vegetation (the Grampians, Southern and North Eastern slopes of Victorian Alps). Translation from the spatial pattern of species diversity to abundance is not straightforward and mapping spatial risk of *Culicoides* abundance is not yet done. However, the maximum abundance values we have by species reach neither the levels nor the duration that have been recorded, for example, for *C. brevitarsis* at Grafton in NSW (19).
- A very positive outcome of the project was the links built up with partners including farmers and offices of DEPI Victoria, providing a network of potential collection sites and collaborators for fieldwork. Technical collaborations with Dr Glenn Bellis (AQIS Darwin) and Dr David Gopurenko (DPI NSW) have also been crucial for several aspects of the project related to taxonomy and behavioural studies.

5.1.2 Host preferences

Urine-baited traps

The technique we employed for baited traps has been used previously for tabanid flies and mosquitoes but was new for *Culicoides*. The results are interesting but puzzling. The relative abundance was difficult to analyse due to the high day-to-day variance so we focused on the distributions of sex and parous status among the different species and baits. We also conducted experiments on *C. brevitarsis* at Beatrice Hill as it was considered that this may serve as a useful positive control for attraction to cattle urine due to its dung-breeding and cattle-biting behaviours. This was the first time that a choice between cattle and sheep has

been offered to *C. brevitarsis* in a relatively unbiased approach. Although we found more *C. brevitarsis* were attracted to cattle urine than to water and to sheep urine, the results were not statistically significant. However, the sex ratio was not distorted at all by cattle urine. This could be due to swarming formations by which males and females gather before mating. There was no difference between young and aged females in attraction to the baits and water, although the difference approached significance for sheep urine. One hypothesis could be that *C. brevitarsis* have a genetic predisposition for attraction to cattle (may be less for sheep), whatever the age or the sex: young female and male, for swarming and females as a whole, young or not, for biting and egg-laying.

For the other species, there was a clear reluctance for males to go towards cattle urine. This was driven mainly by *C. austropalpalis* values but it was also significant for *C. marksi* in the NT and approached significance (lower numbers) in Victoria. Concerning sheep urine, male *C. austropalpalis* demonstrated a behaviour significantly different in the NT (where sheep are absent and where the sex-ratio was comparable in the three arms of the test) and in Victoria (where experiments were run on sheep farms and where the sex-ratio was the lowest for sheep urine). This appears to be evidence of selected behaviour by environment. For *C. marksi*, young females were more repulsed by cattle urine than the aged ones, significantly in Victoria but not significantly in the NT. This was the same pattern as recorded for *C. austropalpalis*. For these two species, no influence on age of female collected is noted for sheep urine. For *C. victoriae*, collection numbers for were low in the NT and so the behaviour could only be observed in Victoria: the proportion of older females was higher for both cattle and sheep urine than for water baits. Finally, these patterns could be explained by low or no attraction of these species to cattle or sheep due to the absence of genetic selection, with aged females attracted for biting through environmental printing.

Molecular analysis of blood meals

Previous experiments on blood meal analysis were performed in NSW and the NT using precipitin tests (11, 12). Our data are very comparable to these with the important exception that we observed that the blood meals of *C. victoriae* and *C. bundyensis* were of marsupial orign. Even though numbers were low, it was very intringuing that most of the samples of various species from Victoria fed exclusively on marsupials. Human blood was detected in some samples and this may have been due to laboratory contamination during preparation for PCR. However, it is also possible that these results are true as the field experiments exposed the worker to bites and there was a significant correlation between samples that tested positive for human blood and *Culicoides* species which were found with blood from exotic placental mammals rather than endemic fauna (marsupials and birds). Blood meal analysis revealed a clear distinction between the *Avariatia* (mainly exotic) and *Marksomyia* subgenera which fed upon exotic and large placental mammals, and *C. victoriae* group and *C. austropalpalis*, both endemic and having fed on endemic marsupials and birds. This appears to be a strong phylogenetic signal.

5.1.3 Vector competence

Experiments were conducted to test for vector competence for BTV serotype1, the most abundant serotype circulating in NSW. The molecular methods we employed allowed testing individual midges rather than in batches and up-scaling to test more than 400 midges. The feeding rates and survival rates were comparable to the series of experiments conducted in

Europe by Carpenter (20). Among the tested *Culicoides*, 7% gave positive results for the presence of viral RNA following feeding. However, after a fresh blood meal the Ct values were higher than the starting point of the experiment. In most of the midges tested viral RNA levels were undetectable and, amongst the positives, less RNA was present after 12 days incubation, indicating an absence of viral replication. *Culex annulirostris* mosquitoes were used as control. As expected, this showed an absence of replication following an infective blood meal but signs of replication following direct microinjection in the thorax, bypassing the midgut barrier.

Our work generated new data on *C. austropalpalis*, one of the most abundant *Culicoides* species in Australia. This species has not been properly tested for vector competence due to difficulty of such experiments and its bird-feeding behaviour which relegated it to a low priority. BTV has never been isolated from *C. austropalpalis*. Although Kay and Standfast (15) referred to vector competence studies for bluetongue, there are no details of methods or numbers of samples tested. On the other hand, isolations of Warrego virus (orbivirus circulating in marsupials), Facey's Paddock (Simbu group bunyavirus) and Kununurra (ungrouped rhabdovirus) (21), and Wallal virus RNA (22)(orbivirus, responsible for kangaroo blindness) have been reported from the species. In South Africa, isolations of BTV have been made from bird-feeding *Culicoides* species(23).

5.1.4 Risk analysis

We conducted an assessment of risk of bluetongue transmission presented by each of the main species currently present in Victoria. This was based primarily on quantitative data generated by our work and previously published work to compare these species with known vectors. The most abundant species, *C. austropalpalis*, does not appear to present risks to the Victorian sheep or cattle industries. Another important species, *C. marksi*, despite it abundance and taste for cattle, could be also excluded as a transmssion risk by vector competence studies. The situation with *C. victoriae* and *C. bundyensis* is more complex. Each can be at relatively high abundance. Although their primary hosts are marsupials, published work has shown each has fed on cattle and sheep in New South Wales. Experimental infection data do exist for *C. bundyensis* and are reassuring. However, these tests were conducted in the NT and it is highly probable that the taxon present in Victoria is a different species. Furthermore, *C. victoriae*, as a complex of species, could present different levels of vector competence and no data exist on this question. Superficially, this indicates a need for multiple assessments of different populations which occur together in a highly seasonal demographic pattern. However, several taxa of this group were associated with saline or estuarine environments and present characteristics of autogeny and so could be considered of minor or no importance in a bluetongue transmission cycle.

One of the major recognised risks in Victoria is the potential for migration of *C. brevitarsis* to southern regions. The assessment of this risk was out of the scope of his project. However, it is notable that, despite a high sampling effort, we did not capture any specimen of *C. brevitarsis* in Victoria, even in peripheral zones such as the Wodonga region or Gippsland.

5.2 Practical implications for industry

5.2.1 Is the sheep industry fully safe in Victoria?

Obviously, the answer to this question is not straightforward. Several elements have to be considered:

Among the positive elements:

- No bluetongue disease outbreak has occurred in the past in Victoria, Tasmania or South Australia.
- The southern limit of the major vector, *C. brevitarsis*, currently does not reach the Victorian border.
- The current distribution of *Culicoides* species has not recently shifted dramatically.
    - The seasonal abundance peak is relatively short during the hot and dry period and lasts up to April.
- The most abundant species in Victoria (*C. austropalpalis*) is not considered to be a potential vector for bluetongue serotype 1, the most abundant serotype in NSW.
- The *Culicoides* species present in Victoria are endemic and their primary host species are very probably endemic to Australia (marsupials, birds).
- Several stakeholders in the sector (veterinarians, DEPI officers and farmers) have been very open to collaboration and interested in the project outcomes.

Some elements are more problematic:

- The project shows that some zones in Victoria could present relatively high abundances of *Culicoides* (Benalla region, Grampians, Gippsland). These abundance zones could be very restricted and fine mapping is difficult and not yet accomplished.
- Despite their Australian origin, some of the abundant *Culicoides* species have been described having bitten cattle and sheep.
- The *C. victoriae* group is very diverse and the vector competence by individual taxon is not known.
- A very high number of samples to test for viral challenge is needed to fully exclude a species as vector.
- The dynamics of evolutionary adaptation of BTV to the *Culicoides* species in Victoria remains undetermined.

Nevertheless, the situation of the southern zones of Australia is better known compared to the situation in Europe prior to the 2006 bluetongue serotype 8 epizootic. We know which species are present and the range of hosts and degrees of vector competence for several endemic species. We have also established molecular tools to untangle the cryptic species. Definitively, the sheep and cattle industries are in much better place than Europe was in 2006.

5.3 Unanswered questions / additional research recommended

5.3.1 Taxonomy and vector competence of *C. victoriae* complex

We have molecular data and morphological vouchers which will allow us to define more precisely the taxonomy of this group. This will mean describing the new species and re-describing *C. victoriae* from Cockatoo, the original location from where it was described by McFie in 1941. Any new work on this species and related species should start from this core species.

Assessing the vector competence of cryptic species which are sympatric (living at same time in the same environment) is a difficult task. Some of the sites we have sampled could allow the collection of relatively high numbers of individuals. However, the short season of peak abundance and the variability between years are limiting. Technically, we have set up the tools for molecular diagnostics of species and set up the conditions for individual viral RNA testing. This could be used as backbone for a more complete assessment of vector competencies.

5.3.2 Trophic deviation

We have shown that primary hosts of *C. victoriae* and several endemic species are marsupials. However *C. victoriae* and *C. bundyensis* have been described having bitten cattle and sheep. This behaviour is not uncommon in biting insects, as a strict narrow range of hosts could be very detrimental to a species. However, faced with primary and alternative hosts, the primary host would the one of choice. This raises the possibility that that a new method of protection for cattle and sheep could be to deliberately favour the maintenance of primary host populations (birds for *C. austropalpalis*, marsupials for *C. victoriae*) in farm environments. This technique is known and applied for malaria vectors, termed as zooprophylaxis, by putting cattle inside the village in the immediate vicinity of human dwellings to encourage the malaria vectors to bite cows rather than humans. The impact of this method on bluetongue potential transmission is not known. Our observations show that *C. victoriae* and *C. bundyensis*, in the presence of sheep, has fed on marsupials but this needs a more quantitative assessment. It would also be important to consider potential side effects such as pathogen spill-over from marsupials to livestock as these *Culicoides* could be considered as bridge-vectors for potential pathogens between reservoir and naïve mammal populations. However, it is likely that the long history of grazing in southern Australia would have already resulted in such spill-overs if they were indeed possible.

5.3.3 Extrapolation to other Australian states

The extrapolation of the data on Victoria to other states is not possible. Fewer species are described from the other states in the South. It seems reasonable that Tasmania (island, smaller area) would have far fewer species than Victoria. In the southern parts of Western Australia and South Australia, the number of known species is probably underestimated and their biology and dynamics are underexplored.

5.3.4 Bluetongue virus serotypes

We have tested only BTV-1 in this study as it is the most frequently isolated serotype in New South Wales. Although there are currently 11 known BTV serotypes in northern Australia, the east coast (NSW and QLD) appears to function as a bottleneck for transmission of most

serotypes from the north and beyond Australian borders. Only BTV-1, BTV-21, and more recently BTV-2 and BTV-15, have reached eastern Australia to date and this bottleneck may be providing relative protection for sheep and cattle these regions. We do not know the mechanisms by which this bottleneck is acting (e.g., climate, vectors, viruses, cattle, grazing or environmental conditions) but one of the most logical explanations is the association between vector competence and BTV serotype/viral segment specificity. Our preliminary data using next generation sequencing has shown that BTV genome segment diversity was very limited in QLD and NSW until the recent introductions of BTV-2 and BTV-15 which have now recombined by segment reassortment with BTV-1 and BTV 21. Given the impact that destabilisation of this screen could have on the industry, it is very important to studying the mechanisms underlying the bottleneck. This would allow us to forecast the risks of potential intrusion of new BTV strains, including the highly pathogenic ones (such as serotype 8), and develop more informative surveillance tools.

5.3.5 Population genetics of *Culicoides* species. Endemic species.

We have assessed the potential for local *Culicoides* to support BTV transmission in Victoria. Although we currently assess the risks as relatively low, we have shown it is very difficult to rule out any risk of local transmission or an associated disease outbreak. Indeed an important question which has not been studied is the potential for a local outbreak in NSW to spread to Victoria or the neighbouring temperate zones through southern migration of existing vector species, reflecting the spread of *C. imicola* from North Africa and the Middle-East into southern Europe. Modelling of passive wind-borne displacement has shown the relative frequency of exchange of *Culicoides* between Indonesia and Australia and our work on population genetics of *C. brevitarsis* (Onyango et al, in review) demonstrate a high levels of population flux accross the Australian continent. Understanding how the populations of southern endemic *Culicoides* species are linked together could help us to forecast the rate of spread of midge-borne virus throughout the entire temperate region. We have developed a new methodology for such an assessment by using the next generation sequencing tools. We applied this method to *C. brevitarsis* (see above) and *C. imicola*, vector of bluetongue and African horse sickness in Africa (Onyango et al, in prep). Translating this method to the local endemic species is relatively easy and affordable and could reinforce the position of livestock industry in term of viral safeguard for their temperate zones.

5.4 What could have been improved in the project delivery

5.4.1 Sampling in the field

The choice of the sampling sites was directed to the regions of importance for both cattle and sheep. However, the choice of properties and farms on which we have worked was more difficult and so a random sampling was not possible. Given the scale of the study, it is difficult to be certain that our sites are fully representative of all the farming ecosystems in the state. For that we have complemented our sampling in parks and reserves. A more systematic sampling, in conjunction with DEPI and/or professional associations could lead to a more complete view of the *Culicoides* populations in Victoria.

5.4.2 Human ressources

The project had the benefit of the participation of a talented and motivated technician. Several volunteers have also helped in sampling in farm environment. However, after the techncian’s sick leave then retirement, administrative difficulties led to delays in delivering

outputs. Still now, the technician position has not been filled and these delays have not been fully absorbed and the publication of the data has been partly postponed.

5.4.3 Links with professional associations

Very quickly during the project, contacts with DEPI have been very usefull. Our partners have developed a strong interest and have been involved in the field work. This occurred not only at local levels but also central levels of DEPI have been interested. Feedback has been provided with several meetings but a general feedback at the end of the project is still to take place. No link has been established with other professional associations in a systematic way.

5.5 Meeting the specific objectives of the project

5.5.1 Survey of *Culicoides* species in Victoria, particularly those that occur in grazing country and are associated with cattle and sheep

During the study, we set traps for *Culicoides* in Victorian farms, with cross- and repeated transversal samplings. This has provided an updated view on *Culicoides* in Victoria, particularly those occurring in farm environments. Elements of their biology and population dynamics have been established. We have developed molecular tools to decipher the taxonomic postion of several species.

5.5.2 Determine the host feeding preference of potential vectors

We have developed a new technique for assessing the host attraction preference of *Culicoides* species. Complementarily, molecular analysis of blood meals has given data on the post-feeding phase.

5.5.3 Determine the competence of potential vector species for transmission of Australian BTV serotypes

We have tested the most abundant *Culicoides* species found in Victorian farms in experimental viral challenge with the most frequently isolate of the three BTV serotypes that occur in New South Wales. Individual testing of the midge has been performed by qPCR and controles have been tested.

5.5.4 Assess the risks of BTV transmission in southern Australia

We have performed an analysis of the risks presented by the most prevalent species in Victoria from the data generated by the project and from previously published work.

5.5.5 Success in achieving milestones

Each step of the project has been assessed by milestone report. We present here the last milestone, as the last milestone report and the final report are merged together.

Quantitative data on vector competence of the primary candidate species occurring in the southern Australia:

Experimental infections of *Culicoides* have been made from wild caught midges (Benalla) in Feb-April 2014. Most of them were *C. austropalpalis*, the most abundant midge in Victorian farms. Among 416 infected midges which have survived for 12-13 days (25% survival rate)

and been tested by quantitative PCR for bluetongue virus, 31 (7.4%) gave a positive signal and 47 (11%) an inconclusive signal. However the positive CT values were all higher than CT at start of the experiment. This means there is neither sign of RNA nor virus replication in any of the tested midges. As control, microinjected mosquitoes showed RNA replication.

A relative risk analysis for transmission of bluetongue virus in sheep and cattle in Victoria with respect to the host feeding preferences and vector capacity of potential vectors.

Analysis of vector threat with our current data and published papers or reports shows that *C. marksi*, if competent, would have been a problem in Victoria. However both the absence of bluetongue virus isolation from this species and the experimental infection data published with high number of tested flies testify for a safe status. *C. victoriae* present a high parous rate and there are published reports of blood meals taken on bovids. However, our local captures showed marsupials as major source of blood meals and the experimental infections led by Muller in 1993, despite the moderate number of tested midges, give evidence for a low risk for this group. However, a firm conclusion is hampered and postponed by the taxonomy of this group of sibling species. Indeed, closely related species and taxa sympatric in several zones of Victoria make the experiments of infection particularly difficult to implement. Lastly, *C. austropalpalis,* by far the most abundant species found in Victoria, despite it abundance and a higher parous rate in Victoria than in the NT, has an affinity for birds and has been shown not to support replication of bluetongue virus..

Final Workshop performed

Face to face meetings with farmer partners (Benalla, Glenaladale) were held in 2015. A session with Victoria DEPI will be held in November to present the major outcomes of the project. The final technical report will be distributed to all the partners.

Two papers submitted to a peer review journal with one already accepted

One paper has been submitted and accepted about *Culicoides* in Victoria and assessment of bacterial symbionts: Novel detection of low level *Cardinium* and *Wolbachia* infections in *Culicoides* PT Mee, AR Weeks, PJ Walker, AA Hoffmann, JB Duchemin, submitted to Applied and Environmental Microbiology.

Three other papers are in progress:

- Distribution of *Culicoides* species in Victoria, including *C. victoriae* group molecular diagnostic and distributions modelling: to be submitted to *Parasites and Vectors*).
- Bluetongue transmission risk in temperate regions of Australia: the importance of Host-culicoides relationships: to be submitted to *Parasites and Vectors,* presented as poster at Roma, Nov2014.
- Bluetongue transmission in the southern parts of Australia: lack of evidence of vector competence for the most abundant species: to be submitted to *Veterinaria Italiana*.

The project data have been presented in several meetings:

- *Culicoides* in Victoria – reassessment of the bluetongue transmission risk in southern zones of Australia: oral communication at the Arbovirus research in Australia and Mosquito Control Association Symposium Surfers Paradise, Sep 2012

- *Culicoides* in Victoria – reassessment of the bluetongue transmission risk in southern zones of Australia: oral communication at EIDS meeting Geelong, Oct 2012
- Bluetongue transmission risk in temperate regions of Australia: the importance of Host-*Culicoides* relationships: poster at Bluetongue and related Orbivirus IV International Conference Roma, Nov 2014
- Bluetongue transmission risk: Comparison of Europe and Australia contexts, Third Forum of French Researchers in Australia Network Sydney, Nov 2014

6 Conclusions/recommendations

6.1 Future R&D

6.1.1 Research on Southern *Culicoides*

To define the vector competence of *C. victoriae* group species in the Southern zones of Australia

6.1.2 Research on virus

To understand the bottleneck in BTV populations between North and East episystems

6.1.3 Farm environment

To assess the protective role to cattle and sheep of the endemic fauna, marsupials and birds in farm ecosystems.

6.2 Practical activities

6.2.1 Review of NAMP sampling sites in Victoria

To target the zones where the *Culicoides* are the most abundant

To target the zones of incursions of species coming from New South Wales

7 Key messages

7.1 The situation of the temperate zones of Australia is very particular

What is known about the bluetongue transmission in northern tropical areas of Australia is not immediately translatable to the Southern temperate zones.

7.1.1 Beyond the climate

Most of the *Culicoides* biting midges present in the Victoria are completely different from those in tropical areas, including the nortern-eastern coast of New South Wales. All of them are endemic and come from a local ancestral stock. We have not detected a recent change in the *Culicoides* species present in Victoria. Some specimens of new species coming from Northern states were detected but they are rare and did not shows signs of a new deep colonisation.

7.1.2 The vectors and their hosts

The origin of the Victorian *Culicoides* makes them very different as species but also in their behaviours. The northern species which are exotic midges that bite large placental mammals which they use as vectors. The southern species use and prefer to bite marsupials and birds. However, this is not an absolute rule and most of these species could bite alternative hosts such as cattle and sheep.

7.1.3 The risk

Some zones of Victoria are prone to high abundance of *Culicoides*. Most of these *Culicoides* species are not able to transmit BTV . Certain zones such as southern estuarine areas could present very high *Culicoides* populations but they appear to be safe for sheep industry. However, we are not able to rule out a minor risk of BTV transmission or an outbreak in Victoria. This risk is very probably low and smaller than in northern zones. The current situation is comparable to that 20 years ago but we cannot extrapolate to the incursion of new virus strains or the southern movement of vectors from the North. However, we are now in a better position than Europe was before the massive outbreak which commenced in 2006.

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9 Appendix

9.1 Data tables

9.1.1 List of taxa collected during our sampling

In bold “Yes”, new species for Victoria, already existing in neighbouring states. In bold” New”, new taxa to be described (Bellis and Duchemin works in progress)

Taxonomy	Species (wing reference in wing atlas)	Collected Yes/No/New
Jacobsoni Complex	*Avaritia sp N2 - (10) subgenus*	Yes
Antennalis gp	*antennalis*	Yes
Antennalis gp	*antennalis gp sp1 (65)*	No
Bancrofti group	*bancrofti*	Yes

Clavipalpis gp	*bunrooensis*	**Yes**
Marksomyia	*dycei*	Yes
Marksomyia	*marksi*	Yes
Marksomyia	*parvimaculatus*	Yes
Molestus group	*balhorni = molestus gp2 (153)*	**New**
Molestus group	*molestus gp3 (154)*	Not found
Molestus group	*subimmaculatus*	Yes
Ornatus group	*marmoratus*	Yes
Ornatus group	*ornatus gp (180?)*	**Yes**
Shermani group	*rabauli*	Yes
Victoriae group	*bundyensis*	Yes
Victoriae group	*bundyensis-like*	**New**
Victoriae group	*fulbrighti*	Yes
Victoriae group	*mcmillani (232)*	Not found
Victoriae group	*multimaculatus*	Yes
Victoriae group	*victoriae*	Yes
Victoriae group	*victoriae gp15(238)*	Not found
Victoriae group	*Victoriae-like*	**New**
Victoriae group	*Victoriae-like*	**New**
Victoriae group	*Victoriae-like*	**New**
Victoriae group	*waringi*	**Yes**
Williwilli group	*austropalpalis*	Yes
Williwilli group	*sigmoidus*	Yes
Williwilli group	*williwilli*	**Yes**
Williwilli group	*williwilli gp18 (281)*	Not found

9.1.2 Ecological indices by site

Site	Abundance	Richness	Shannon
Benalla	7521	13	1.027
Breamlea	3	2	0.636
Cockatoo	14	2	0.257
Colac	56	1	0
Darwin	**5809**	**20**	**1.734**
Emu Farm	2286	8	0.451
Geelong	697	1	0
Glenaladale	1003	7	1.418
Lal-Lal	2	1	0
Newnerella	111	3	0.793
Orbost	2000	1	0
Port-Fairy	4	2	0.693
Snowy River Wildernest	47	3	0.205
Stratford / Avon	38	3	0.243
Wodonga	1072	9	1.217
Woodside	1	1	0
Grampians	1761	5	1.377
Wilsons Promontory	50	2	0.098
Otways Horden Vale	1	1	0
Myrtleford	23	2	0.295
Little Desert	1794	3	0.664

9.1.3 Average mean *Culicoides* caught by Green LED traps by site in Victoria (NT's site sin bold as a control)

Site	Trap count	Average total *Culicoides*	Std. Dev.
Beatrice Hill	**11**	**510.9**	**236.6**
Benalla	52	191.8	497.0
Breamlea	4	0.8	1.5
Cockatoo	2	7.0	7.1
Colac	5	11.2	25.0
Darwin urban	**9**	**23.0**	**20.5**
Emu Farm	11	201.3	292.9
Geelong	26	26.3	26.2
Glenaladale	18	56.4	95.3
Grampians	3	549.0	497.6
Lal-Lal	6	0.7	1.0
Little Desert NP	3	604.3	825.1
Mortlake	10	0.2	0.6
Myrtleford	1	23.0	
Newnerella	1	110.0	
Orbost	1	2000.0	
Otways Horden Vale	1	1.0	
Port-Fairy	7	0.7	1.5
Snowy River	2	23.5	17.7
Stratford / Avon	1	38.0	
Wilsons Prom.	1	50.0	
Wodonga	14	76.6	139.4
Woodside	2	0.5	0.7

9.1.4 Distribution of species

Taxonomy	Species	N
Jacobsoni Complex	*Avaritia sp N2 - (10) subgenus*	1
Antennalis gp	*antennalis*	2
Bancrofti group	*bancrofti*	2
Clavipalpis gp	*bunrooensis*	7
Marksomyia	*dycei*	206
Marksomyia	*marksi*	889
Marksomyia	*parvimaculatus*	2
Molestus group	*balhorni = molestus gp2 (153)*	2050
Molestus group	*subimmaculatus*	1
Ornatus group	*marmoratus*	10
Ornatus group	*ornatus gp (180?)*	27
Shermani group	*rabauli*	4
Victoriae group	*bundyensis*	**751**
Victoriae group	*bundyensis-like*	
Victoriae group	*fulbrighti*	5
Victoriae group	*multimaculatus*	36
Victoriae group	*victoriae*	**3369**
Victoriae group	*Victoriae-like*	
Victoriae group	*Victoriae-like*	
Victoriae group	*Victoriae-like*	881
Victoriae group	*waringi*	112
Williwilli group	*austropalpalis*	8393
Williwilli group	*sigmoidus*	4
Williwilli group	*williwilli*	6
	Undetermined	76

9.1.5 Abdomen status by species in NT and Victoria

Darwin	**Pigmented**	**Bloodfed**	**Gravid**	**EmptyClear**	**Total**	**parous rate**
actoni/minimus	129	24	14	163	330	50.6%
austropalpalis	109	57	238	625	1029	39.3%
brevitarsis	221	21	490	355	1087	67.3%
flumineus	64	0	1	0	65	**100.0%**
histrio	10	7	3	17	37	54.1%
marksi	293	54	28	336	711	52.7%
ornatus gp#6	192	44	129	374	739	49.4%
oxystoma	23	5	0	72	100	28.0%
peregrinus	13	2	3	43	61	29.5%

Victoria	**Pigmented**	**Bloodfed**	**Gravid**	**EmptyClear**	**Total**	**parous rate**
austropalpalis	1074	75	911	2216	4276	48.2%
balhorni	0	0	48	1	49	**98.0%**
bundyensis-like	78	29	121	109	337	67.7%
dycei	9	4	78	11	102	89.2%
marksi	76	6	233	129	444	70.9%
multimaculatus	2	0	32	1	35	**97.1%**
ornatus gp#6	8	1	4	14	27	48.1%
victoriae-like	365	50	910	372	1697	78.1%
victoriae-like2	0	0	367	14	381	**96.3%**
waringi	0	0	45	10	55	**81.8%**