Vector competence of *Culicoides* for arboviruses: three major periods of research, their influence on current studies and future directions

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Summary
The spectacular and unprecedented outbreaks of bluetongue virus (BTV) that have occurred in Europe since 1998 have led to increased interest in those factors that determine competence of *Culicoides* biting midges (Diptera: Ceratopogonidae) for arboviruses. In this review the authors critically examine three major periods of research into the biological transmission by *Culicoides* of two economically important arboviruses of the family Reoviridae: African horse sickness virus (AHSV) and BTV. First they examine early studies, largely conducted in southern Africa, that played a key role in initially implicating *Culicoides* as agents of AHSV and BTV transmission. Then they examine advances in understanding made following the establishment of colonies of the BTV vector species *Culicoides sonorensis*, which have largely shaped our current understanding of BTV and AHSV transmission. They then consider attempts in recent years to implicate vectors of BTV in the European Union during what has become the most economically damaging series of outbreaks in recorded history. In some cases the origin of these outbreaks was uncertain and unexpected, particularly in northern Europe, where BTV had not previously occurred. Limitations imposed on studies of vector competence by the biology of *Culicoides* are then discussed, along with advances in the technologies now available and the logistics of working upon agents requiring biosecure containment outside their endemic range. Finally, the authors suggest areas that have either been poorly addressed to date or entirely ignored and ways in which studies could be conducted to provide standardised data for comparison worldwide.

Keywords

Introduction

*Culicoides* biting midges (Diptera: Ceratopogonidae) are vectors of a wide array of pathogens, including arboviruses of international importance in the worldwide production and trade of livestock (1, 2, 3). Globally, the most important of these, at present, is bluetongue virus (BTV), which can replicate in all ruminant species examined to date and is the aetiological agent of the haemorrhagic disease bluetongue (BT). Multiple and persistent incursions of BTV strains into Europe continue to have a huge socioeconomic impact, both directly through clinical BT and indirectly through those livestock movement restrictions employed to limit BTV spread (3, 4, 5, 6, 7). *Culicoides* also act as vectors of the virus that causes African horse sickness (AHS), a highly lethal disease of horses. Mechanisation has reduced the global significance of AHS virus (AHSV) in transport and industry. However, the horseracing industry, which retains
an important economic role in many countries, continues to be affected and the traumatic nature of AHS also inflicts an uncharacterised social impact on companion animal owners. Similarities between BTV and AHSV in epidemiology have also led to this virus consistently appearing in many horizon-scanning risk assessments of arbovirus incursion in Europe (8, 9, 10, 11, 12, 13).

In this review the authors examine three periods of research that have helped shape our understanding of the role of Culicoides as vectors of BTV and AHSV. In exploring this area, they also illustrate some of the challenges faced by researchers attempting to define field and laboratory vector competence. Firstly, they review the studies that led to the initial implication of Culicoides as vectors, carried out primarily at the Onderstepoort Veterinary Institute (OVI) in the Republic of South Africa during the 1930s and 1940s. These studies were instrumental in shaping the future programme of work carried out globally on Culicoides and remain among the best known and most commonly cited in the field. The authors then consider the colonisation of the BTV vector Culicoides variipennis (later renamed Culicoides sonorensis) in Texas during the late 1950s. This, together with parallel advances in diagnostic assays for BTV, enabled the wide range of studies in the United States (USA) and the United Kingdom (UK) that have largely defined our understanding of vector competence in the genus. Lastly, they critically explore the recent outbreaks of BTV in Europe since 1998 and include an account of the integration of modern diagnostic techniques into studies of vector competence in Culicoides. Potential future advances in technology that may facilitate a clearer understanding of the role of Culicoides as vectors are then discussed, as is the ongoing requirement for parallel progress in identifying, rearing and maintaining epidemiologically relevant species.

Strictly speaking, vector competence does not include the broader ecological aspects of vectorial capacity (e.g. vector survival, biting rates; see below), although those concerns clearly have influenced the choice of species studied for their competence. Vector competence is defined as the ability of a vector to biologically transmit arboviruses between susceptible hosts (mechanical methods of transmission are addressed elsewhere in this publication). To achieve this, the arbovirus must infect, replicate, and disseminate within the vector and must reach secondary organs, including the salivary glands. As vectors are poikilothermic, the vector and must reach secondary organs, including the arbovirus must infect, replicate, and disseminate within the vector. While superficially simple, vector competence is increasingly viewed as a complex trait, involving genetic determinants in vector, virus and host (driven by co-evolution) and modulated by a vast range of variables. Our knowledge of these areas has expanded greatly in recent years. This is due, in part, to the advent of genomic techniques. Informative reviews that have the potential to revolutionise our understanding of this area have already been published concerning these developments, including considerations of insect immunity (14, 15).

No attempt is made in this review to examine non-viral animal pathogens transmitted by Culicoides (16), or pathogens of humans transmitted by this genus (17). There is also no detailed discussion of either the epidemiology of specific arboviruses (2, 3, 4, 6, 7) or Culicoides biology and ecology (1, 18, 19), for which a large number of reviews are already available. To fully understand the detailed background to studies in this area, the reader is referred to broader reviews that provide wide-ranging examinations of the role of Culicoides as vectors (10, 20, 21, 22).

Early studies of Culicoides as vectors of arboviruses

The studies that first implicated Culicoides biting midges as agents of arbovirus transmission were part of a diverse range of studies carried out upon AHSV and BTV at the OVI in the 1930s and 1940s (23). While both viruses were prominent targets for veterinary research in southern Africa at the turn of the 20th Century, AHS in particular had long been viewed as one of the most devastating diseases present in the region since the first introduction of horses from Europe into southern Africa during the 1650s (24). The founding of OVI outside Pretoria on 8 October 1908 allowed the director, Sir Arnold Theiler, who had a career-long interest in AHSV, to target studies towards the design of vaccines for AHSV (25). The Institute was in close proximity to areas with a high prevalence of AHSV (and BTV), which allowed a coherent programme of research on both AHSV and BTV to be developed for the first time. During the years that followed, a vast array of fundamental advances were made in understanding their aetiology (for further details see Erasmus et al. [23]).

By the early 1900s, driven in part by advances in understanding vector-borne diseases, there was increasing suspicion that AHSV and BTV were arboviruses (26). The involvement of night-active arthropod vectors in transmission of AHSV and BTV was supported by a wealth of anecdotal evidence, including the association of epidemics with the latter half of the summer rainy season and low-lying areas, the demonstration that the agents were not directly contagious between livestock and the observation that winter frosts and stabling of livestock at night reduced the probability of infection dramatically (27, 28, 29). Despite this, experimental transmission of BTV and AHSV using arthropods was not systematically attempted until the early 1930s, following a wide range of largely
anecdotal studies that had failed to clearly implicate any specific vector group (30).

Initially, mosquitoes were targeted as the most likely vector group for AHVS and BTV. This was due to preliminary field surveys conducted at OVI and to the documented ability of mosquitoes to transmit a wide range of other important medical and veterinary pathogens (31). In addition, the transmission routes for AHVS and BTV appeared to be similar to the transmission route described in the highly influential studies conducted by the US Army Yellow Fever Commission in Cuba. The first large-scale transmission studies for both AHVS and BTV were carried out during the summers of 1931–1932 (30, 32) and 1932–1933 (33). Taken together, these three reports encompassed over 115 inter-related transmission experiments in sheep and horses using locally collected mosquitoes, primarily from the Aedes, Culex and Anopheles genera. Attempts to infect these mosquitoes centred upon first feeding them upon viraemic donor hosts that had been infected by inoculation either with blood taken from reported cases in the field or from viruses maintained via serial passage in natural hosts or suckling mice. Groups of mosquitoes that had successfully fed were then selected and incubated for varying amounts of time (12 hours to several weeks). Detection of virus was then conducted by grinding these mosquitoes in serum and inoculating the pools into susceptible hosts, then re-feeding live mosquitoes on recipient hosts by either introducing them into a cage containing a host or releasing them into a tent containing a host (the latter being performed in the AHVS experiments only). Due to a lack of diagnostic techniques, evidence of transmission was derived solely from clinical signs in recipient animals and the results of subsequent testing of immunity to further infection following inoculation with strains of BTV or AHVS. Where suitable clinical signs were recorded in recipient hosts, blood samples were also, in some cases, inoculated into further hosts to confirm the presence of virus.

The results of these studies were largely disappointing, with only seven inoculations of putatively infected, incubated mosquitoes leading to the observation of clinical signs. Of these, two inoculations (one of BTV and one of AHVS) contained mosquito pools that had recently fed on the viraemic donor and hence still contained live virus from the original blood meal. Attempts to transmit AHVS or BTV by re-feeding mosquitoes failed entirely, despite using a total of 1,324 individuals of various species. In the case of BTV these results could be called into question either on the basis of previous host infection with the virus (which could not at that time be accurately assessed) or because of limited manifestation of clinical signs (32). The lack of timely, clinically apparent and repeatable responses recorded for AHVS-positive inoculations, however, led to the conclusion that if natural transmission of AHVS did indeed occur through mosquito vectors, this was a rare event and hence could not fully explain endemic circulation of the virus (33).

Following the elimination of mosquitoes as primary vectors of BTV and AHVS, and also due to the increasing availability of relatively effective live attenuated vaccines, there was a hiatus in research in this area until almost eight years later, when OVI began implementing light/suction trapping using a modified New Jersey light trap (34, 35). While details of the abundance of vector groups found in these cases were not published, there is a high probability that these catches were dominated by Culicoides, despite their not having been recorded during earlier surveys using animal-baited traps at OVI and beyond (31, 33). The use of effective and relatively powerful light/suction traps at OVI was key in enabling the collection of large numbers of insects overnight from the field. These could be rapidly sorted into vector groups, homogenised and subsequently inoculated directly into hosts. These studies were also greatly facilitated by the huge abundance of Culicoides species, and especially C. imicola, which exploited the predominantly black cotton soils present in the Onderstepoort area as larval habitats.

Transmission experiments using Culicoides commenced in June 1942 following a very similar design to those conducted with mosquitoes (although certain aspects of the methodology were usually not described in detail). From an unknown number of inoculations of vector groups trapped at night, three apparent cases of BTV from pools of Culicoides were observed in recipient sheep (including one fatality). In addition, an inoculation of field-collected Culicoides in March 1943 also resulted in a clinical case of AHVS and all of these positive results produced clinical disease when sub-inoculated from clinically affected animals into further susceptible recipient hosts. Transmission of circulating viruses was also attempted by directly feeding light-trap-collected Culicoides on naive hosts 23 times for AHVS and 15 times for BTV. Finally, Culicoides were also fed upon BTV-viraemic donor sheep, incubated and then re-fed upon naive sheep. Clinical disease was recorded in a single recipient host using an unknown number of Culicoides at ten days post-feeding on a viraemic animal. From the total number of Culicoides fed, only three individuals were recovered following re-feeding upon the recipient host, of which two had engorged, and these were tentatively identified as C. pallidipennis (later renamed C. imicola).

These early studies of transmission of BTV and AHVS by mosquitoes and Culicoides dwarf any transmission experiments conducted in later studies; however, from a modern perspective they are challenging to interpret. This is because there was a lack of effective diagnostic technology available at that time, the methodological details are either poorly described or omitted, and the highly interrelated experimental designs used are complex. The primary technical importance of the studies rests largely
C. bolitinos is more susceptible to infection with BTV than subsequent oral susceptibility studies seem to indicate that may have been included in these experimental transmissions. Species group; they are morphologically similar and isolation from field populations in specimens screened during Onderstepoort may have also included low numbers (<1%) of virus in mammalian cell cultures, the competence of C. imicola for BTV (37, 38) and AHV (39) is relatively low and feeding responses can vary (40). The low vector competence of C. imicola in the laboratory is supported by low levels of virus in attempts to transmit BTV between sheep, C. brevitarsis on a heroic scale in Australia used 77,000 field-collected EHDV (52), and naïve sheep (36). These, and later studies which demonstrated that C. sonorensis could transmit EHDV, were enabled by the relatively high rate of transmission by established colony lines. In marked contrast, later studies on a heroic scale in Australia used 77,000 field-collected C. brevitarsis in attempts to transmit BTV between sheep, but failed entirely (53), illustrating the difficulties in working with low-competence field populations.

The development of colonies of C. sonorensis as a colony line was the prelude to a wide diversity of studies of vector competence that included defining methods for intra-thoracic infection with BTV and containment during infection studies (54). Increasingly, serial passage of viruses in embryonated hens' eggs was used to improve the logistical flexibility of studies, a process that was later to lead to the routine use of cell-line passaged strains. In an extension of this system, intrathoracically inoculated C. sonorensis fed on embryonated hens' eggs were used as a model for transmission (55) and time-series profiles of EIP virogenesis in infected pools provided the first BTV replication profiles in the insect host (56). Since then, C. sonorensis has acted as the standard laboratory model for Culicoides infection studies worldwide, and the vast range of studies accomplished with this species has been reviewed in detail elsewhere (2, 22, 51).

Development of Culicoides colonies in the United States

It is now widely accepted that the OVI studies provided the first evidence of transmission of AHV and BTV by Culicoides, but at the time the issues described above raised doubts in the entomological community that this was the primary route. Universal acceptance only occurred following further transmission experiments in the USA. Bluetongue virus had initially been identified as an emerging virus in the USA during the 1950s, through clinical cases of what was termed 'soremuzzle' recorded in Texas range sheep during 1948 (42). The virus was subsequently isolated from sheep in California during 1952 (43) and wide-scale surveys during the 1970s confirmed that BTV was circulating endemically, mainly in the western and southern sections of the country (44, 45, 46). How and when BTV and the closely related epizootic haemorrhagic disease virus (EHDV) were first introduced into the Americas remains unknown, although evidence of clinical signs consistent with diseases caused by these viruses had been observed anecdotal in sheep by stockhandlers for many years prior to eventual confirmation (47). In direct contrast to the earlier studies carried out at OVI, where initial studies were conducted with mosquitoes, studies in the USA were quick to focus on Culicoides fauna in the years following the discovery of BTV. Light/suction trap surveys were soon underway in the region and they identified the C. variipennis taxonomic complex as dominating farm-associated habitats (48).

Morphological studies identified five subspecies within this complex (49), of which three were later given full species status (50). Two of these, now known as C. variipennis and C. sonorensis, reached high abundance through exploiting organically enriched environments as larval habitats. A challenge faced by researchers lay in consistently separating these species morphologically. Early papers examining vector competence refer only to C. variipennis. Fortunately, in nearly all cases, the species involved can be identified retrospectively as C. sonorensis due to the much greater prevalence and abundance of this species in the western states of the USA.
Current research on responses to infection relies substantially on this single species due to its unique biology among BTV vectors (2, 57). This lack of diversity in laboratory hosts has led to the use of cell cultures derived from C. sonorensis as a proxy for infection in the insect host in a wide range of fundamental studies examining immunological responses (51). Laboratory infection trials with BTV have already demonstrated that bottlenecks in establishment of colony lines of C. sonorensis could mean that the susceptibility of these insects is unrepresentative of the susceptibility of field-collected individuals from the same location and could lead to a reduction in the heterogeneity of response (58). These are minor issues, however, in comparison to the wider question of comparability of vector competence mechanisms in C. sonorensis with those in other, generally uncolonised, Culicoides species worldwide.

It would be expected that the fundamental process of infection and dissemination of arboviruses within the Culicoides host is conserved across the genus, as it closely resembles that found in other Dipteran vectors of arboviruses. Following the imbibing of a blood meal from a viraemic host, infection in C. sonorensis is thought to occur via attachment of BTV to the luminal surface of microvilli lining the hind mid-gut prior to formation of the peritrophic matrix (59, 60, 61). Following entry and replication within the cells of these microvilli, progeny virus escapes through the basolateral surface into the haemocoel and infects secondary organs, including the salivary glands (60, 61). Transovarial transmission of BTV through infection of ovaries has not been recorded for C. sonorensis under laboratory conditions, as has been discussed elsewhere (22). Replication of BTV in the salivary glands is followed by release into the salivary ducts and subsequent transmission when the vector next bites a susceptible host (20). Important mid-gut infection barriers and mid-gut release barriers have been described for BTV transmission (59, 60, 62), but an interesting apparent divergence from some mosquito-borne models of arbovirus transmission is the lack of salivary gland barriers to infection. This has been observed repeatedly in C. sonorensis (63, 64, 65), and was also later recorded in C. imicola (66) and some northern European species (67), although results with C. brevitarsis in Australia were equivocal (68). The answer to the key question of whether or not this observation is an artefact of intrathoracic inoculation is not yet known, as our understanding of the process of arbovirus infection of salivary glands is incomplete in most vector systems.

Due to its flexibility as a laboratory model, C. sonorensis was identified at an early stage as an excellent subject for studies of trait inheritance (62). Pioneering selective breeding experiments suggested that a genetic mechanism existed for vector competence through maternal inheritance modulated by paternal imprinting (2, 14, 69). It is clear, however, that vector competence in Culicoides can also be modulated according to arbovirus species and strain (70). High larval rearing temperature (>32°C) (1, 71) and physical disruption of the mid-gut by filarial infection (72) can also influence competence substantially, as can saliva-arbovirus interactions (73). Microbiome-vector competence interactions are also likely to be influential and while these studies remain in their infancy in Culicoides research, they are currently revolutionising how we view mosquito-arbovirus interactions (74).

The degree to which these mechanisms of vector competence in C. sonorensis are representative of other vector Culicoides species is unknown (14) and has the potential to vary at a seasonal, population and species level. Modern technologies of transcriptomics and genomics are now being applied to inheritance of vector competence in C. sonorensis and the first de novo whole-genome sequencing is being carried out for this species (51). This is likely to facilitate a clearer understanding of vector competence in C. sonorensis. A major future challenge will lie in conducting comparative studies on less malleable species in other regions and drawing conclusions regarding the impact of any differences on the likelihood of arbovirus transmission. The ongoing uncertainty in this area is best illustrated by the recent outbreaks of BTV in Europe. They have highlighted our inability to accurately predict global arbovirus movements, except in the broadest sense.

**Emergence of bluetongue virus in Europe**

The unprecedented incursions and persistence of multiple BTV strains into Europe began in 1998 and initiated a third major period of research on vector competence of Culicoides, centred upon the detection of novel vector species. Prior to 1998, there was already evidence that Culicoides species present across Europe were capable of transmitting BTV and AHFV (75, 76). Of the species implicated by these studies, C. imicola was suggested to be by far the most important in transmission of both arboviruses. This was due to correlation in distribution of outbreaks with abundant populations of this species (which in Europe was restricted to the Mediterranean Basin), a greater number of positive isolations of arboviruses being made from C. imicola collected from outbreak sites and the convincing implication of the species in transmission in Africa (22). Bluetongue virus and AHFV were also isolated, however, from other species of Culicoides with a pan-European distribution and these were suggested to represent a risk for northwards movement of arboviruses (77).

Prior to the BTV outbreaks, the most widely distributed and abundant species in the Palaearctic region were those classified within the Avaritia subgenus, namely C. obsoletus;
C. scoticus, C. dewulfi, C. chiopterus and C. montanus. With the exception of the rare and geographically restricted C. montanus, males of these species could be accurately differentiated on the basis of the morphology of the ninth sternite (78, 79), but few males are collected in light/suction trap surveys. In contrast, female Culicoides are almost always collected in far greater numbers at light in proximity of livestock than males, and are challenging to reliably separate to species level, particularly in the case of C. obsoletus and C. scoticus. The geographic range of C. obsoletus and C. scoticus was known to overlap with C. imicola (and hence it is probable that these species were the source of the isolations of BTV in Cyprus and AHSV in Spain), while the more northern areas of distribution of C. dewulfi and C. chiopterus precluded their initial southern European involvement. Several other species were also known to be present on farms across Europe in smaller numbers, including C. pulicaris, C. punctatus and C. impunctatus, but the vector potential of these species was largely unknown.

In addition to isolation of arboviruses from field populations of Palaearctic Culicoides species in southern Europe, artificial infection experiments were also conducted using membrane-based blood-feeding systems in the UK in the 1980s (67). Feeding rates during these and subsequent infection experiments using this range of species were poor, illustrating another fundamental impediment to testing vector competence in the laboratory. Small-scale infection studies of pools potentially containing C. obsoletus, C. scoticus, C. dewulfi and C. chiopterus from a population in southern England, however, demonstrated a low level of vector competence when fed on a viraemic sheep infected with a BTV-4 strain (77). While experimental details are scant, this early study remains the only recorded experiment to infect northern European species of Culicoides with arboviruses using a viraemic host.

From 1998 to 2006 detection of arboviruses in field populations of Culicoides and artificial infection in the laboratory were based around virus isolation (e.g. cell culture) and the technical limitations of these assays have been reviewed (6). Briefly, a key requirement for vector implication, where transmission is not conducted between live hosts, is the ability to determine full dissemination of arboviruses within the arthropod, including infection of the salivary glands, enabling onwards transmission (21, 80). In Culicoides the issue of assessing dissemination within the insect is simplified by an apparent lack of salivary gland infection and escape barriers; in the absence of these, the detection of an arbovirus in secondary organs and the haemocoeel is likely to be an indication that the infection is fully disseminated (65). Determining true levels of susceptibility in field populations of Culicoides remains challenging, however, as the proportion of competent individuals is often low and persistent infection of the mid-gut is common in refractory individuals (60, 64). Studies in the UK using a colony line of C. sonorensis from the USA demonstrated that individuals with sub-transmissible infections could be separated from fully disseminated individuals using quantification (60, 80). Culicoides sonorensis containing greater than $10^{2.5}$ tissue culture infectious doses (TCID$_{50}$) of BTV using a 50% endpoint of cytopathic effect on baby hamster kidney (BHK-21) cells were found to be fully infected, whereas those not exceeding this quantity were not (60). While this threshold is likely to vary according to species of Culicoides and the diagnostic procedure used to quantify the arbovirus within the putative vector, this discovery had the wider effect of emphasising the importance of quantification.

The trials conducted in southern Europe following the incursion of multiple BTV strains between 1998 and 2006 provided convincing evidence that Culicoides in the Palaearctic region were capable of transmission, but failed to provide a comparative measure of competence between species (12). In field studies, undifferentiated pools of C. obsoletus and C. scoticus collected at BTV outbreak sites in Italy were identified as being infected with BTV (81, 82). A key limitation of field-based studies of this time was that detection of BTV in Culicoides pools required isolation through adaptation to passage in embryonated hens’ eggs, this made accurate quantification of virus in the first sample impossible, as strains varied widely in their initial cytopathic impact on eggs. Still, the fact that isolations of BTV were made from Culicoides without any visible blood meal remnants implies at least one individual infection in each positive pool, even if it is difficult to entirely discount the presence of individuals infected at a sub-transmissible level (22, 65).

Simultaneously, studies in the UK used artificial infection methods and a BTV-9 strain originating from Kosovo and discovered differential susceptibility to infection across undifferentiated populations of C. obsoletus, C. scoticus, C. dewulfi and C. chiopterus (83). The studies used an infection technique (direct feeding on blood-virus mixtures not imbibed through a membrane) that was known to underestimate competence in Culicoides (84) and a virus isolate that had been passaged in tissue culture. Peak titres in susceptible individuals were as high as $10^8$ TCID$_{50}$, which was considered to be indicative of fully disseminated infection. In addition, the populations examined during the study varied widely in competence and it was suggested that this might be due to variation in competence between multiple species included in pools (83). Hence, while it was clear that species other than C. imicola had a high potential to transmit BTV in Europe, the role each species played was as yet unclear.

In 2006, BTV (a BTV-8 strain) entered northern Europe for the first time in recorded history (6). This event coincided
with the development and implementation of real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays as a front-line diagnostic tool across Europe. Implementation of this tool in vector implication was to prove challenging. Initial studies conducted on field populations in Germany (85) and the Netherlands (86, 87) processed pools of Culicoides from the field, but failed to provide any measure of quantification for positive pools (12) and were therefore uninterpretable. Fully disseminated individuals could not be separated from pools containing Culicoides with sub-transmissible infections; pools potentially also contained individuals that had cleared the virus but retained inactivated remnants of BTV RNA in the gut (12). Following these trials, semi-quantitative measures of BTV load (C \text{ t} values) were provided in two larger studies, one in Germany (12) and the other in Belgium (88), which were therefore uninterpretable. Fully disseminated populations in Germany (85) and the Netherlands (86, 87) were therefore uninterpretable. Fully disseminated viruses but retained inactivated remnants of BTV RNA in the gut (12). Following these trials, semi-quantitative measures of BTV load (C \text{ t} values) were provided in two larger studies in Belgium (88) and Germany (89). The study conducted in Germany is particularly notable for illustrating the high-throughput processing power that roboticised rRT-PCR systems provide, screening 24,513 pools of Culicoides for BTV and finding 585 positive pools. While convincingly high loads of BTV RNA, indicative of at least sub-transmissible infection, were found in some of these pools, vector species diagnostics remained problematic.

A major development in this period was the increasing use of molecular markers to construct phylogenies within European Culicoides species and to provide tools to determine which species a female individual belonged to; previously it had been hard to separate females to species level (90, 91, 92, 93, 94, 95). In the same field study in Germany (89), species presence/absence in pools was determined by the use of a multiplex assay based on the Cytochrome Oxidase-1 mitochondrial marker (90). This assay had only been standardised for processing single individuals, and the competitive performance of amplification for each primer set had not been assessed (65). In addition, the high likelihood of there being a single infected Culicoides in each positive pool also raised the question of sensitivity of detection for each species within pools, which was also not addressed. These methods were more fully integrated into laboratory infection studies in the UK, albeit with vastly smaller sample sizes (96). A low-passage strain of BTV-8 was used and replication of BTV to potentially transmissible levels was identified in both C. obsoletus and C. scoticus (96). Optimal homogenisation of Culicoides in this study was enabled by the prior development of standard programmes for a TissueLyser (97).

Recent outbreaks of Schmallenberg virus (SBV), a novel orthobunyavirus of ruminants discovered in Europe in 2011 (98), have seen methodologies for determining vector competence in field populations of Culicoides revisited. In a ground-breaking study conducted in the Netherlands, screening for competent individuals was carried out using small pools of decapitated heads, with the bodies for each pool retained in individual storage (99). Where pools tested positive for SBV RNA using rRT-PCR, the remaining carcasses from the pools were tested to determine how many individuals were infected. In addition, DNA extracted from the SBV-positive carcasses was also sequenced using the nuclear 18S Internal Transcribed Spacer 1 (ITS-1) 5.8s marker (100). This has provided matching haplotype-competence data and convincingly demonstrated infections in C. obsoletus, C. scoticus and C. chiopterus. Laboratory trials with C. sonorensis subsequently demonstrated that at least some of these infections provided convincing evidence of a role in transmission of SBV by defining semi-quantitative measures of RNA in specimens with fully disseminated infections (64). Subsequent studies conducted in Belgium additionally implicated C. dewulfi in SBV transmission (101, 102), and in France, a single pool of C. nubeculosus was also identified as positive for SBV (103), confirming an earlier study using the UK colony line (64).

While initial studies of SBV suggested that C. scoticus had a greater role as a vector of SBV than C. obsoletus (99), later studies have identified greater numbers of infections in C. obsoletus (101). This highlights a major challenge in interpretation of field competence results: the numbers of positive individuals identified almost always constitute a very small proportion of the total Culicoides population. An example from Australia is the trapping of Culicoides in the Northern Territories, where only a single isolate of BTV was obtained from processing over 170,000 individuals in pools. In the vast majority of cases, fine-scale differences in competence are best approached using standardised comparison of infection rates in the laboratory. It is important that similar studies for SBV be conducted, as they will help to determine species involvement in the transmission and subsequent spread of the virus and enable a comparison with BTV-8.

Lessons from vector competence testing of Culicoides and future directions

Vector competence studies require a sound taxonomic base

Taxonomic studies of Culicoides worldwide have been recently reviewed (104). Correct identification to species level of specimens processed during vector competence assessments is of paramount importance. This is because morphologically similar species can vary widely in their vector competence (and vector capacity) and this can influence arbovirus distribution. The general lack of taxonomic expertise has hampered efforts to distinguish between species; only a few highly specialised experts are able to achieve accurate morphological separation. However, studies in this area are
being revolutionised by the sequencing of molecular markers with standardised quality parameters – most notably through the Barcode of Life Initiative (105) – and by improvements in access to conventional taxonomic knowledge thanks to the worldwide availability of open-access taxonomy resources on the Internet.

There is currently great potential to establish global initiatives to trace the phylogenetics of Culicoides as a means of understanding the evolution of their role as vectors of arboviruses. A secondary focus of this research would lie in resolving relationships between haplotypes in widely distributed species (e.g. C. obsoletus), as this may influence the period of co-evolution between potential vectors and endemically circulating arboviruses. The rationale for conducting these studies would be to provide standardised estimates of putative vector diversity (with a high probability of revealing the existence of cryptic species), but also to provide a target for detailed comparative studies of competence mechanisms. The application of genomic and transcriptomic techniques within the genus is likely to become increasingly commonplace and a fundamental understanding of the taxonomy of the species under investigation will be a vital component in accurately targeting these studies, at least in the early stages of research. Such studies are likely to be extremely challenging, particularly where laboratory colonies of species cannot be established. However, by clarifying the functional constraints that vector/arbovirus co-evolution imposes on competence, these studies represent the best hope for predicting shifts in the distribution of arboviruses in the future.

**Vector competence is a single component of vector capacity**

A major consideration in risk assessments of arbovirus incursion is that vector competence is just one component of vector capacity. The degree of vector competence, perhaps surprisingly, may in some scenarios not be as influential as other vectorial capacity traits. This is exemplified by C. brevitarsis in Australia, which is not very susceptible to BTV infection, but is still a major BTV transmitter due to the vast populations that develop in close proximity to livestock. An array of livestock-associated Culicoides species has been tested for competence in South Africa, where laboratory-based testing has been employed for many years. Some species are susceptible to infection, including some that are present around livestock in only small numbers and perhaps do not even feed on mammals. A major future challenge will be interpreting the epidemiological impact of such species. When a primary suspect vector and form of transmission is established as a risk, as was the case historically with C. imicola in the Mediterranean Basin, C. sonorensis in the USA and C. imicola in South Africa, we tend to concentrate research on them, even at the expense of investigating other possible vectors and transmission routes. We need to consider the risk of movement of arboviruses between overlapping populations of vectors using a more comprehensive approach. In addition, other forms of transmission (e.g. mechanical, non-viraemic or contact) tend to receive relatively little attention.

**Techniques to test vector competence evolve rapidly and vary in their standardisation**

Taking into account the vast geographical distribution of some species (e.g. C. imicola and C. obsoletus), it can be asked to what degree vector competence studies, most often based on relatively few individuals from single geographical populations, can be extrapolated from the laboratory to the field? A major point of contention in studies of laboratory-based infections of Culicoides, particularly in areas outside regions of endemic arbovirus circulation, has been the use of cell-adapted strains for infection. The use of such strains reflects logistical limitations of feeding Culicoides under containment and the fact that colonies do not exist for most species. For example, it is quite difficult to time restricted periods of transmissible viraemia in hosts with significant day-to-day variation in field availability of Culicoides for infection. Unsurprisingly, use of highly passaged strains has been most prevalent in Europe, not only because these strains require costly biosecure containment, but because the availability of putative vectors is restricted in this region owing to the short adult vector season, which is only a few months long, and the unpredictability of day-to-day flight activity. Another reason that the use of cell-adapted strains has been more widespread in Europe than elsewhere is that, as the region that has been most affected by BTV in recent years, it has received more research funding for these studies than any other region. More recently, studies are increasingly using passage in C. sonorensis cell lines as a means of detecting and maintaining arboviruses prior to use in experiments. While closer to a ‘natural’ system than techniques used previously, the concern still remains that these processes reduce the comparability or realism of studies and hence research to investigate their impact should be prioritised.

More widely, however, it is clear that future studies will increasingly use next-generation sequencing (NGS) to conduct screening of field populations of Culicoides for arboviruses. This is likely to revolutionise our understanding of the diversity of pathogenic and non-pathogenic Culicoides-borne viruses of livestock, wildlife and humans circulating in ecosystems. In addition, in the longer term, these techniques will enable vastly more efficient and cost-effective screening for potential pathogens at a relatively low overall cost. This is likely to enable the re-establishment of research in endemic regions where Culicoides-borne arboviruses are currently of low national priority, but of great relevance to understanding worldwide transmission. Standardisation of such techniques and the implementation of pipelines for storage and analysis of bioinformatic data
are both likely to present major challenges; however, these are already in the process of development for mosquito-borne pathogens (106, 107, 108).

**Virus-related factors remain poorly understood as a driver of vector competence**

While vector-related mechanisms of competence have been investigated in *C. sonorensis*, there have been no systematic attempts to understand shifts in the geographic distribution of strains according to adaptation to novel vector species. There is now unequivocal evidence that certain BTV strains have moved from areas dominated by sub-Saharan species of Culicoides to those dominated by northern European species. A key question, however, is whether these species have always possessed the capability of transmitting BTV. It is possible that the unprecedented number of BTV incursions into Europe in recent years solely reflects increased frequency of introduction, although the numerous potential routes of entry make this hypothesis difficult to test (109). Additional factors increasing the efficiency of transmission could be climate mediated (3) or result from shifts in the competence phenotype of the vector population in areas of introduction.

A complementary theory could be that certain BTV strains have emerged through an ability to adapt more easily to injection and dissemination within Palaearctic vector species. At first, it was suggested that the apparent stability of circulating strains in the USA and Australia was due to Culicoides–BTV co-evolution. However, in these cases, the majority of surveillance was conducted through identification of serotype and therefore it potentially underestimated strain diversity and shifts of distribution in these areas. There is also a major issue in areas using vaccines containing live attenuated virus that has in some cases been found to be transmissible by Culicoides. This may confound detection of field strains and influence competence rates in populations which, when tested, were only identified to serotype level (110). Laboratory studies have demonstrated that colony lines of *C. sonorensis* can be infected with and replicate a wide range of arbovirus strains from outside their endemic range (70, 111, 112). It remains unclear whether this is influenced by selection imposed by the colonisation of *C. sonorensis* or the adaptation of arboviruses to passage in vertebrates or cell culture. This area should be investigated fully using standardised experiments across regions supporting different primary Culicoides vectors as it could substantially improve our understanding of BTV and AHHSV epidemiology and probability of spread.

**Vector competence is not solely a consequence of arbovirus–vector interactions**

Studies of mosquito–arbovirus interactions are currently focusing on the impact of bacterial endosymbionts on vector competence, particularly in limiting virus replication (113, 114). While this area has not been addressed in detail in Culicoides, replication of BTV in *Drosophila melanogaster* is known to be inhibited by infection with the wMel strain of *Wolbachia pipientis* (115). There is not yet direct evidence that members of the microbiome influence arbovirus infection in Culicoides; however, NGS technologies will in the future allow communities to be dissected in detail for the first time. Although logistically challenging, establishing selected microbes in field populations will enable a community ecology approach to examining these relationships and may also provide practical methods for control.

There are also other possible mechanisms influencing vector competence under laboratory conditions that require confirmation in the field. Co-infection with filaria and arboviruses has been cited as a potential means of vector competence enhancement (116), but has never been surveyed on a wide scale in the field. High temperatures, particularly during larval development (71), may also enhance competence, but that area has also not been sufficiently addressed. It still needs to be determined if a single individual female will be able to become infected with, and replicate, more than one closely related virus, e.g. two or more strains of BTV, or BTV and AHHSV, simultaneously. This will especially be of importance in endemic areas where these viruses are co-circulating. These are just some of the factors that may influence vector competence. They require further study, as they have the potential to confound investigations of vector competence. A better understanding of what influences competence may also provide the means to increase the accuracy of future risk assessments of pathogen incursion and spread.

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Compétence vectorielle des *Culicoides* vis-à-vis des arbovirus : les trois grandes phases de la recherche, leur influence sur les études actuelles et les perspectives d’avenir

S. Carpenter, E. Veronesi, B. Mullens & G. Venter

Résumé
Les foyers spectaculaires et sans précédent de fièvre catarrhale ovine (FCO) enregistrés en Europe depuis 1998 ont suscité un regain d’intérêt pour les facteurs déterminant la compétence des moucherons piqueurs *Culicoides* (Diptera : Ceratopogonidae) vis-à-vis des arbovirus. Les auteurs font une analyse critique des trois grandes phases de la recherche sur la transmission biologique par les *Culicoides* de deux maladies aux conséquences économiques considérables, causées par des arbovirus de la famille des *Reoviridae*, à savoir le virus de la peste équine et le virus de la FCO. Ils rappellent tout d’abord les études les plus anciennes, conduites pour la plupart en Afrique australe, qui ont permis d’attribuer aux *Culicoides* le rôle d’agents de la transmission des virus de la peste équine et de la FCO. Ils retracent ensuite les progrès des connaissances suite à l’établissement de colonies de l’espèce *Culicoides sonorensis*, vecteur du virus de la FCO, qui ont fortement contribué à la compréhension que nous avons aujourd’hui de la transmission des virus de la peste équine et de la FCO. Ils décrivent enfin les récentes tentatives d’identification des vecteurs du virus de la FCO dans l’Union européenne, qui a connu les vagues d’infections les plus lourdes de conséquences au plan économique jamais enregistrées. Dans certains cas, l’origine des foyers était imprécise et inattendue, en particulier en Europe du Nord où le virus de la FCO n’avait jamais été observé auparavant. Après avoir examiné les contraintes que la biologie des *Culicoides* impose aux études sur la compétence vectorielle, les auteurs font le point sur les avancées technologiques ainsi que sur les conditions logistiques de confinement et de sécurité biologique qu’il convient de respecter lorsque l’on travaille avec des agents biologiques en dehors des aires d’endémie. Enfin, ils mentionnent certains aspects négligés ou totalement ignorés par les chercheurs et font quelques propositions pour que la recherche puisse fournir des données standardisées en vue de leur comparaison au niveau mondial.

Mots-clés
Competencia de *Culicoides* como vector de arbovirus: tres grandes periodos de investigación, su influencia en los estudios actuales y futuras líneas de trabajo

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**Resumen**

Los espectaculares e inauditos brotes de infección por el virus de la lengua azul (VLA) que se han producido en Europa desde 1998 han suscitado un mayor interés por los factores que determinan la competencia del jéjén *Culicoides* (Diptera: Ceratopogonidae) como vector de los arbovirus. Los autores proceden a un examen crítico de los tres grandes periodos que han marcado las investigaciones sobre la transmisión biológica por *Culicoides* de dos arbovirus económicamente importantes de la familia *Reoviridae*: el virus de la peste equina (VPE) y el VLA. En primer lugar pasan revista a los primeros estudios, realizados sobre todo en el África meridional, que sirvieron para empezar a identificar al género *Culicoides* como agente de transmisión del VPE y el VLA. Después explican cómo se logró una creciente comprensión del fenómeno gracias al establecimiento de colonias de la especie vectora del VLA *Culicoides sonorensis*, comprensión que ha influido sobremanera en lo que hoy sabemos y entendemos de la transmisión del VLA y el VPE. A continuación describen los intentos realizados en los últimos años para descubrir los vectores del VLA en la Unión Europea, en el curso de lo que ha sido la serie de brotes económicamente más nociva de la que se tiene constancia histórica. En algunos casos el origen de esos brotes resultaba incierto o inesperado, especialmente en el norte de Europa, donde nunca antes se había manifestado el VLA. Los autores examinan después las limitaciones que la biología de *Culicoides* impone a los estudios de su competencia como vector, así como los avances tecnológicos ahora existentes y las consideraciones logísticas que se derivan del hecho de trabajar con agentes que requieren medidas de contención y seguridad biológica fuera de sus zonas de endemicidad. Por último, los autores apuntan una serie de temas hasta la fecha poco estudiados o completamente desatendidos y sugieren fórmulas para llevar a cabo estudios que permitan obtener datos normalizados con fines de comparación a escala mundial.

**Palabras clave**


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**References**


