

The prevalence of *Culicoides* spp. in 3 geographic areas of South Africa

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Culicoides midges,
Over-wintering,
South Africa.

Summary

The seasonal abundance of *Culicoides* midges, the vector of Bluetongue and African horse sickness viruses (BTV/AHSV) and the presence of viruses in midges were determined in 3 geographic areas in South Africa. In the Onderstepoort area, more than 500,000 *Culicoides* midges belonging to 27 species were collected. Eighteen midge species were collected throughout Winter and the presence of AHSV and BTV RNA in midges was detected using real time reverse transcription quantitative polymerase chain reaction. The nucleic acid of AHSV was found in 12 pools out of total pools of 35 *Culicoides*. Twenty-five *Culicoides* species were detected in the Mnisi area. The RNA of BTV was detected in 75.9% of the midge pools collected during Winter and 51.2% of those collected during Autumn. Antibodies for BTV were detected in 95% of cattle sampled using a competitive enzyme-linked immunosorbent assay (cELISA). The dominant species in these 2 areas was *Culicoides imicola*. Eight *Culicoides* species were collected in Namaqualand. *Culicoides imicola* represented the 0.9% and *Culicoides bolitinos* the 1.5% of total catches, respectively. Antibodies for AHSV were detected in 4.4% of 874 equines tested using an indirect ELISA. Results showed that transmission of AHSV and BTV can carry on throughout Winter and the outbreak may begin as soon as *Culicoides* populations reach a certain critical level.

Culicoides spp. in tre aree geografiche del Sudafrica

Parole chiave

Culicoides spp.,
Moscerino,
Sudafrica,
Svernamento,
Virus della Bluetongue,
Virus della Peste equina
africana.

Riassunto

I *Culicoides* sono noti vettori del virus della Bluetongue (BTV) e del virus della Peste equina africana (AHSV). Il lavoro riporta i risultati sull'abbondanza stagionale di *Culicoides* e sulla presenza di BTV e AHSV in diversi vettori, in tre aree geografiche del Sudafrica. Nell'area di Onderstepoort sono stati individuati più di 500.000 esemplari di *Culicoides* appartenenti a 27 specie diverse. Durante la stagione invernale sono state individuate 18 specie. Frammenti di RNA di AHSV e BTV sono stati rilevati mediante specifiche RT-qPCR. L'RNA del virus AHSV è stato individuato in 12 pool di *Culicoides* su 35 esaminati. Nell'area di Mnisi sono state identificate 25 specie di *Culicoides*. L'RNA di BTV è stato rilevato nel 75,9% dei pool di *Culicoides* catturati durante la stagione invernale e nel 51,2% di quelli catturati durante la stagione autunnale. Anticorpi contro BTV sono stati osservati nel 95% dei sieri prelevati da bovini dell'area di Mnisi ed esaminati con il metodo c-ELISA. La specie dominante in queste due aree è risultata *Culicoides imicola*. Nella regione di Namaqualand sono state individuate otto specie, *C. imicola* ha rappresentato lo 0,9% delle specie catturate e *Culicoides bolitinos* l'1,5%. Anticorpi contro il virus AHSV sono stati individuati mediante ELISA nel 4,4% degli 874 equini esaminati. I risultati ottenuti hanno dimostrato che in Sudafrica la trasmissione di BTV e AHSV può continuare durante la stagione invernale mentre, con ogni probabilità, si ha la comparsa dei primi focolai quando le diverse popolazioni di *Culicoides* raggiungono un livello riproduttivo critico.

Introduction

Bluetongue (BT) and African horse sickness (AHS) are endemic diseases and have a strong seasonal appearance in South Africa (SA). The viruses causing these diseases, Bluetongue virus (BTV) and African horse sickness virus (AHSV), are double-stranded RNA viruses in the genus *Orbivirus*, family *Reoviridae* (Mertens *et al.* 2008).

The substantial economic losses caused by BT are due to its high morbidity and sometimes high mortality, particularly in certain breeds of sheep (e.g. Merino), as well as to its associated impact on livestock industries. This is a result of international regulations and embargoes on trade to restrict the movement of livestock and their germplasm (MacLachlan and Osburn 2006). Losses in non-endemic regions can be high, Belgium, for example, having lost a quarter of its sheep population during an outbreak caused by BTV serotype 8 in North-Eastern Europe during 2007 (Gloster *et al.* 2008).

Although the economic impact of AHS has not been calculated for SA, the loss of not being able to export horses is currently likely to be many millions of South African Rand. For example, the 2004 outbreak of AHS in the South Western Cape led to an embargo on the export of horses, with a loss estimated to be at least R30 million in foreign exchange during the 24 months following the outbreak (Sinclair *et al.* 2006).

Orbiviruses are biologically transmitted by some species of haematophagous midges belonging to the *Culicoides* genus (Diptera: Ceratopogonidae). Out of the over 1,400 species described worldwide, less than 1% have been implicated in the transmission of BTV and AHSV (Meiswinkel *et al.* 2004). The distribution of these diseases is not only dependent on the availability of susceptible hosts, it is also closely linked to the distribution and abundance of vector competent midge species and climatic conditions that support large populations of these insects (Mellor *et al.* 2000, Vellema 2008). Based on their relative abundance around livestock, *Culicoides imicola* and, to a lesser extent, *Culicoides bolitinos* are considered the most important vectors of these viruses in SA.

In SA, both BT and AHS have a strong seasonal appearance with most cases reported at the end of Summer and beginning of Autumn, with no cases being reported during the Winter months. The over-wintering mechanism of these viruses is not clear. The viruses may be maintained/survive over-winter either in sub-clinically infected hosts like donkeys and/or zebras (AHSV) or cattle and wild bovines (BTV) as well as in adult or sub-adult *Culicoides* midges. Transovarial transmission also provides a mechanism for virus maintenance and

has been proven for several arboviruses, e.g. Dengue virus in *Aedes* mosquito species (Lee and Rohani 2005). However, transovarial transmission has not yet been demonstrated conclusively for members of the genus *Culicoides* (Nunamaker *et al.* 1990, Nunamaker *et al.* 1997, White *et al.* 2005).

The number of diagnostic samples received at the Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI) (Dr Marco Romito, PCR Unit, ARC-OVI, Personal communication) and at the Virology Section of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria have increased over the last decade. During the 2012/2013 season, cases from Gauteng were already reported in November. The first cases in Mpumalanga, Limpopo, and KwaZulu-Natal Provinces, considered endemic areas for AHS, were only reported in February and March 2013 (Venter *et al.* 2014). In 2014, AHSV was reported from regions in the Eastern Cape as early as August¹.

Despite these outbreaks, it is still generally accepted that seasonal epidemics spread from the Kruger National Park and adjacent parks to South and Westwards (Coetzer and Guthrie 2004). The extent of these annual AHS epidemics is dependent on favourable climatic conditions, which will increase breeding and spread of the vector (Coetzer and Guthrie 2004). This indicates that circulating infected adult midges are present throughout the year. This is the case especially in regions with moderate Winter temperatures as experienced in SA. As a consequence, outbreaks can commence as soon as the midge numbers reach a critical level.

South Africa is characterised by a huge variation of climatic conditions. The climate ranges from tropical to sub-tropical high Summer rainfall areas in the North-Eastern parts of the country, to semi-desert conditions in the North-Western parts. As altitude increases towards the central plateau, there is a corresponding drop in Winter temperatures. Despite these variations in climate, both AHSV and BTV seem to be endemic in all parts of SA. In most parts of the country, climatic conditions are suitable for large numbers of adult *Culicoides* midges to remain active throughout the year and daytime temperatures seldom reach below zero degrees Celsius. It is therefore possible that small populations of virus-infected adult midges survive long enough between outbreaks during mild Winter. To ensure a continuous transmission of these viruses, vector-free periods must be of shorter duration than the maximum period of viraemia in the vertebrate population (Mellor 1994).

¹ http://www.africanhorsesickness.co.za/early_warning_stats.asp.

In order to understand the seasonal abundance of *Culicoides* midges, the presence of viruses in midges and the possible mechanisms of over-wintering of these viruses, midges were collected during different seasons in 3 geographical areas in SA. The prevalence and abundance of species of *Culicoides* midges in the area and the presence of AHSV and/or BTV in the midges were determined. The analysis of the variations of these parameters in different areas will improve our epidemiology understanding of these diseases under variable climatic conditions.

Materials and methods

Collection sites

Onderstepoort (S 25°39', E 28°11'; 1,219 m above sea level) is situated in the Highveld of SA, a high-altitude grassland area and highly urbanised (Figure 1). It is a Summer rainfall area and most precipitation occurs as brief afternoon thunderstorms. However, relative humidity never becomes uncomfortable. Winters are crisp and dry with occasional frost. Livestock farming is limited to small holdings. According to the African horse sickness Trust, the 7.1% (32,714) of the total horse and donkey population (458,321) in SA is in the Gauteng Province². Despite being geographically the smallest province with the lowest equine population, between 13% and 62% of all cases of AHS in SA are reported from Gauteng Province, annually².

Mnisi (S 24°71', E 031°34587' to S 24°57635', E 031°35109' and S 24°68782', E 031°27770' to S 24°62001', E 031°42599') is a rural area in the Mpumalanga Province of SA (Figure 1). Two thirds of the study area is on the interface with provincial and private game reserves and wildlife, which can act as potential reservoir of viruses. The area comprises a savannah ecosystem and has a sub-tropical climate and a Summer rainfall pattern. Livestock farming is the main agricultural activity. It involves indigenous breeds of cattle, goats, and donkeys. Very few sheep are present. Although cattle are considered potential over-wintering hosts for BT, their role in the epidemiology of the disease in South Africa is still unclear (Nevill 1971). Cattle are generally sub-clinically infected and they could be important as maintenance hosts of BTV (Barratt-Boyes and MacLachlan 1995).

Namaqualand is situated in the arid North-West of South Africa. It lies between 16°27' and 19°0' East; 28°0', and 31°10' South and is a Winter rainfall region (Figure 1). It forms a distinct bio-geographical area

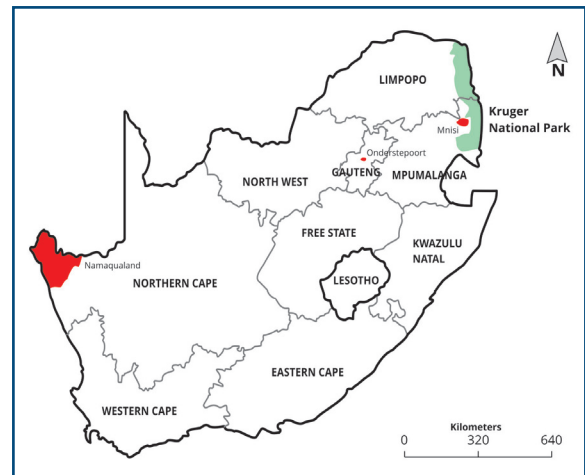


Figure 1. Collection sites of *Culicoides* in different geographical areas of South Africa.

within the larger Succulent Karoo biome (Desmet 2007). Namaqualand is sparsely populated with about 77,000 inhabitants. The main agricultural activity is small stock farming (Desmet 2007).

Sample collection

Culicoides midges were collected using 220 V blacklight Onderstepoort down-draught light traps (Venter *et al.* 2009) operated from 1 hour before sunset to 1 hour after sunrise. Mosquito netting placed around the trap excluded large insects from the collections. To preserve the midges for virus isolation, collections were made directly into phosphate buffered saline with calcium and magnesium (0.05 g/l each) (PBS+) to which 0.5% Savlon® antiseptic (chlorhexidine gluconate 0.3 g/100 ml and cetrimide 3.0 g/100 ml) (Johnson & Johnson, Cape Town, Western Cape, South Africa) was added (Walker and Boreham 1976, Venter *et al.* 2006). The next day in the morning, after the harvest, collections were washed with PBS and the insects were stored in PBS at 4°C until analysed. Based on abdominal pigmentation (Dyce 1969) the females of all species were age-graded into unpigmented (nulliparous), pigmented (parous), gravid or freshly blood-fed. Males were also counted. After being analysed and age graded, the *Culicoides* midges were removed from the PBS+ and stored at -80°C until assayed for the presence of viruses. Sub-sampled collections were identified to species level (Van Ark and Meiswinkel 1992).

Midges (50 to > 3,000 per pool) were macerated in 1,000 µl Eagle Minimum Essential Medium (MEM) [Highveld Biological (Pty) LTD, Johannesburg, Gauteng, South Africa] (without serum) containing a sterile glass bead. After maceration, insect debris was collected by centrifugation at 1,300 g

² African horse sickness (AHS) Trust. 2012. African horse sickness. <http://www.africanhorsesickness.co.za>.

for 1 minute and the supernatants aliquoted into 0.5 ml microcentrifuge tubes. Midge aliquots were tested for the presence of BTV and AHSV using virus isolation and real time reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Onderstepoort

Light traps were placed once a week overnight for 15 months at the ARC-OVI. Weekly collections were made from June 2010 to September 2011, ensuring that at least 2 Winter periods were covered. During Winter (June to August of the following year) the frequency of collection was increased to more than once a week. Traps were set close to 15-20 horses and cattle and sheep were present in the area, at 0.75-1 km from the collection site (Venter *et al.* 2014).

Since animal numbers are low and livestock farming and horses are limited to small-holdings in this area, no sera were collected from animals.

Daily maximum and minimum outside temperatures at the light trap were recorded using a Tinytag Explorer [Gemini Data Loggers (UK) Ltd, Chichester West Sussex, UK].

Mnisi

Culicoides midges were collected every fortnight starting on the 23rd of April 2013 to the 3rd of May 2013 (Autumn) and again from the 17th of June 2013 to the 15th of July 2013 (Winter). Traps were placed near cattle, where possible.

In addition to the midge collections, sera were collected from 1,260 animals representing different cattle breeds, and included males and females of different age groups. Sera were tested for group-specific antibody to BTV.

Meteorological data for the 4 months (April, May, June, and July) were obtained from personnel of the Southern African Wildlife College in the Mnisi area (personal communication).

Namaqualand

Light trap collections (mainly 12 V) were made from the 30th of June 2008 to the 30th of July 2009. The traps were operated once a week at different sites in the area (Schütte 2013). Midges were collected in 200 ml tap water, to which 5 ml of Savlon® antiseptic was added. Since virus isolations were not performed on these samples, the collected midges were stored in 70% alcohol until identified to species level.

In addition to the midge collections, 842 serum samples from equids were collected between August 2009 and February 2010 and tested for a group-specific antibody to AHSV.

The minimum and maximum temperatures during the collection period were recorded.

Antibody detection

A BTV-specific cELISA (Veterinary Medical Research and Diagnostics, Bluetongue Virus Antibody Test Kit, Inc., Pullman, US) was used to detect antibodies specific to BTV in 1,260 serum samples from cattle collected in the Mnisi area.

An AHSV recombinant VP7 indirect ELISA (Maree and Paweska 2005) was used to detect antibodies specific to AHSV in serum samples collected in the Namaqualand area.

Virus detection

Virus detection was done on *Culicoides* collected in the Onderstepoort and Mnisi areas using cell cultures (BHK-21) (only Onderstepoort midges) and the polymerase chain reaction (PCR). Virus isolation using cell cultures was conducted as described by Venter and colleagues (Venter *et al.* 2006). Samples were incubated at 37°C in an incubator containing 5% CO₂ for 10 to 14 days or until cytopathic effect (CPE) was observed. Samples were passaged 3 times on BHK-21 cells.

Pools of homogenised insect samples, representing all catches were tested for the presence of AHSV RNA (Onderstepoort) and BTV RNA (Onderstepoort and Mnisi) using real time RT-qPCR (Quan *et al.* 2010, Van Rijn *et al.* 2012).

Ethical considerations

Materials used in these experiments posed no health risk to researchers and no vertebrate animals were harmed. The projects were approved by the Ethics Committees of the respective institutions. (Project numbers: OVI: OV07/23/C231; UP: V079-12 and V042/09).

Results

Prevalence of *Culicoides* species in 3 geographical areas of South Africa

Onderstepoort

More than 500,000 midges were collected in 88 collections. The monthly mean per night ranged from 67.0 in July 2010 (Winter) to 52,434.6 in March 2011 (Summer) and the overall mean for the period was 5,747.8 midges per night. Relatively high numbers were found throughout Winter

(average 100 midges per night). *Culicoides* midges belonging to 27 species were collected and parous females (females with at least one blood meal and one completed gonotrophic cycle, so they were potentially infected with AHSV/BTV), were present throughout the year (Venter et al. 2014).

The dominant species was *C. imicola* and mean numbers collected per night ranged from 58.5 in July 2011 to 48,650.4 in March 2011. The *C. imicola*'s representation ranged from 96.2% in August 2010 to 74.9% in January 2011, with a mean of 90.8%. The second most abundant species, *Culicoides enderleini*, accounted for 2.3% of the total number and its highest mean number (1,598.6) was collected in March 2011. This species was absent from the collections made in July. *Culicoides nevillei*, at 1.4%, was the third most abundant species (Venter et al. 2014).

In Summer (December and January), *Culicoides* midges belonging to at least 23 species were collected. During Winter (June and July 2011), the number of species present dropped to 10. Despite the lower species distribution, midges belonging to at least 17 species were present throughout Winter. *Culicoides imicola*, were collected throughout Winter and no midge-free period was recorded (Venter et al. 2014).

The maximum temperature measured during this study was 40°C and, in a few nights, the minimum temperature at the collection site was well below freezing point (-4.5°C), with frost occurring.

Mnisi

A total number of 91,659 *Culicoides* midges was collected in 16 light trap collections and 25 *Culicoides* species were detected. Although collections were restricted to Autumn and Winter, the mean collection size (5,729) in the Mnisi area was equal to the one in the Onderstepoort area (5,748), where collections were made throughout the year. During Autumn, 23 species were identified from 16 collections, while in Winter 20 species were identified from the same sites. Some of the species, i.e. *C. dekeyseri* and *C. glabripennis*, were only collected in Winter time. Whereas, *C. engubandei*, *C. kanagai*, *C. milnei* Austen, *Culicoides* sp. 50 and *Culicoides* sp. 54 p/f were absent during Winter. Similar to Onderstepoort, *C. imicola* was the most abundant species during both Autumn (91%) and Winter (75%) from all collections at all sites sampled. During Autumn, *C. nevillei* (4.0%) and *C. enderleini* (1.3%) were the second and the third most abundant species collected, respectively. In Winter collections, *C. bolitinos* (4.4%) was the second most abundant species and *C. nevillei* (3.4%) the third most abundant one (Steyn 2014).

Out of the 25 sampled *Culicoides* species, 13 species

in Autumn and 16 species in Winter, were found to be both abundant and wide-spread in the area. Species collected both in Autumn and in Winter included *C. bolitinos*, *C. enderleini*, *C. exspectator*, *C. imicola*, *C. leucostictus*, *C. neavei*, *C. nevillei*, *C. pycnostictus*, *C. similis*, *Culicoides* sp. 54 d/f, *C. subschultzei*, *C. tropicalis*, and *C. tuttifrutti*. *C. bedfordi*, *C. zuluensis*, and *C. nivosus* were only present during the Winter period (Steyn 2014).

Parous females were present throughout both sampling periods. A total of 19 out of the 25 species yielded parous females collected in Autumn and in Winter.

The average outside temperatures varied between 17.4°C and 20.8°C, with an average rainfall of 0.02-0.05 mm during the 4 months of sample collection. Throughout the sampling months, the relative humidity was between 50% and 87%, with an average wind speed of 2.9 km/h.

Namaqualand

A total of 134 light trap collections were made during the period from the 30th of June 2008 to the 30th of July 2009 and 13,866 midges were collected. The mean collection size (992) was significantly smaller when compared with the other 2 sites.

Although the number of species (27) collected was comparable with the ones of the other 2 sites, the species composition was very different. The 6 most abundant *Culicoides* collected were: *Culicoides ravus* (29.7%), *C. bedfordi* (25.2%), *Culicoides* sp. 89 (9.6%), *C. subschultzei* (7.4%), *C. herero* (7.1%), and *C. nivosus* (6.8%). The first 2 species, *C. ravus* and *C. bedfordi*, constitute the majority (55.0%) of the total collected species. Of the potential AHSV vectors, (*C. imicola*, *C. bolitinos*, *C. bedfordi*, *C. dutoiti*, *C. engubandei*, *C. magnus*, *C. pycnostictus*, *C. zuluensis*, and *C. gulbenkiani*) 5 of them, namely *C. imicola* (0.9% of the total number of midges collected), *C. bolitinos* (1.5%), *C. bedfordi* (25.2%), *C. engubandei* (<0.1%) and *C. pycnostictus* (3.2%), were present in Namaqualand (Schütte 2013).

The maximum day temperatures during Winter average was 26°C and the minimum 7°C, while Summer temperatures average was 30°C. Evening temperatures sometimes dropped below zero and frost occurred.

Antibody detection

Antibodies specific to BTV were detected in 1,206/1,260 (95.7%) of the tested serum samples in the Mnisi area. Antibodies to AHSV were detected in 37/842 (4.4%) equines tested in the Namaqualand area and were found in 18 of the 58 sampled sites.

Virus detection

Virus isolation, using cell cultures, was attempted on all 60 weekly pools collected from the Onderstepoort area. After 3 passages on BHK-21 cells, viable AHSV was recovered only from 4 pools.

African horse sickness nucleic acid was detected in 12 of the 35 pools collected from the Onderstepoort area and assayed with cycle threshold (CT) values ranging between 26.20 and 39.02. Positive pools were found from October and December to middle May.

In the Mnisi area, BTV RNA was detected in 51.2% of the total midge pools collected during Autumn and in 75.9% of the midge pools was collected during Winter. In this study, 91.2% of the midge pools were collected in the Onderstepoort area.

Discussion and conclusions

Although frost can occur in Onderstepoort and Namaqualand, the daily maximum winter temperatures in the 3 collection sites were generally mild (15-25°C). Summer temperatures in the 3 geographic areas are high (> 35°C), with humidity varying from 50-70% in Mnisi to as low as 20% during the day in Namaqualand. In the Mnisi area, temperature fluctuations are small throughout the year and seldom drop below freezing point. During this study there were nights in Onderstepoort where the minimum temperature was well below freezing point (-4.5°C) and heavy frost occurred (Venter *et al.* 2014). Namaqualand is a semi-desert area with very warm days and cold nights. The average annual rainfall for Onderstepoort is 600 mm, is of 900 mm in Mnisi and less than 100 mm per year in Namaqualand (SA Weather Bureau). The effects of meteorological factors such as temperature, wind speed, and humidity on the presence of *Culicoides* biting midges have been reported before (Braverman 1988, Blackwell 1997, Meiswinkel *et al.* 2000). These factors have been shown to influence the distribution and vector competency of *Culicoides* midges and could affect the occurrence and the number of collected midges (Meiswinkel *et al.* 2000, Carpenter *et al.* 2008).

Despite these variations in climatic conditions, *Culicoides* midges were present in all 3 areas. Of even more significance is that midges were present in low numbers during the Winter months in all the 3 collection sites. *Culicoides* midges belonging to 27 species were collected in the Onderstepoort area, of which at least 17 species were present in the collections throughout the Winter months (June to August) (Venter *et al.* 2014). At least 6 of these species, *C. imicola*, *C. magnus*, *C. nevillei*, *C. zuluensis*, *C. bolitinos*, and *C. enderleini*, are known to be orally susceptible to infection with AHSV (Venter *et al.* 2006, Venter and Paweska 2007).

Similar to Onderstepoort, at least 25 *Culicoides* species were identified in Mnisi, with *C. imicola* being the dominant species throughout the study. This species represented the 91% and 75% of midges collected during Autumn and Winter, respectively. *Culicoides bolitinos*, also known to be the vector for orbiviruses, was the second most abundant species (Steyn 2014). *Culicoides nevillei* was the most abundant species during Autumn and, although the vector competence of this midge species for BTV has not been proven, Epizootic haemorrhagic disease virus, a related *Orbivirus*, has previously been isolated from it (Barnard *et al.* 1998).

The abundance of *Culicoides* in the dry Namaqualand was far less than in the North of the country (Onderstepoort). The total number of midges collected over the year was smaller than the number collected during 1 night in the North of the country. Although Namaqualand appears to be an area with a relative low risk of AHS, 5 potential AHSV vectors were present in this region (Schütte 2013).

The over-wintering mechanisms of orbiviruses (BTV and AHSV) have not been clearly demonstrated yet. In the absence of transovarial transmission other over-wintering mechanisms of these viruses have been hypothesised (Nunamaker *et al.* 1997, White *et al.* 2005, Wilson *et al.* 2009). These include sub-clinically infected hosts showing long viraemic periods with no clinical signs, i.e. zebras, donkeys in the case of AHS and cattle (in SA) in the case of BTV, and the circulation of infected female *Culicoides*. Once a competent *Culicoides* female is systemically infected, it will remain infected persistently, and it will be able to transmit virus until it dies (Mellor *et al.* 2000).

Culicoides imicola adults can survive for more than 15 days and some of the species, for example *Culicoides pycnostictus*, can survive up to 54 days at temperatures as low as -1.5°C (Nevill 1971) and adult longevity increases with a decrease in ambient temperature (Wellby *et al.* 1996, Gerry and Mullens 2000, Lysyk and Danyk 2007). Major temperature fluctuations have been shown to decrease the biting rate of *Culicoides* midges (Mellor *et al.* 2000, Tweedle and Mellor 2002). The minimum temperature required for the replication of orbiviruses in the *Culicoides* vector was determined to be between 11°C and 13°C (Carpenter *et al.* 2011). Due to low Winter temperatures, the rate of virus replication and consequently the virus titre in these potentially infected midges have also been low (Wellby *et al.* 1996, Mellor *et al.* 1998, Paweska *et al.* 2002).

Males and gravid females of Nulliparous parous were present in collections from all the 3 sites, in both Autumn and Winter sampling. The percentage of parous females theoretically gives an indication of the rate of transmission and the presence of parous

females in Winter indicates that blood feeding and therefore the potential to transmit BTV/AHSV continues throughout the colder months in the Mnisi and Onderstepoort areas.

The most abundant midge species is not always the most competent vector species in transmitting a specific virus (Standfast *et al.* 1985). Although the vector capacity of less abundant species may be low compared to the one of more widespread and abundant species (Standfast and Dyce 1972), they can still play an important role in the maintenance of these viruses.

The RNA of BTV was detected in 51.2% of the midge pools collected during Autumn and 75.9% in midge pools collected during Winter in the Mnisi area. The seroprevalence of BTV-specific antibodies in cattle was 95.7%. The positive PCR results obtained in midges confirmed the widespread circulation of BTV in Mnisi. Cattle seldom develop clinical BT in SA and their role in the epidemiology of BT is probably the one of an amplifying host in spring (Nevill 1971). Mnisi is predominantly a cattle-wildlife interface and the presence of clinical BT is rarely noticed.

Both AHSV and BTV RNA were detected in a sub-set of midges collected in the Onderstepoort area using real time RT-qPCR. Viral nucleic acid was detected as early as spring (October), which may indicate over-wintering in adults. Although the midges from Namaqualand were not analysed for virus, ELISA results indicate the presence of antibodies to AHSV in 4.4% of 874 analysed equines. Although the virus, as well as the vector, is present in this area no outbreaks of AHS are reported. This means that either there are infrequent introductions of the virus or that there are insufficient vectors or hosts to

maintain an epidemic (Schütte 2013). It was noted that outbreaks of AHS do occur regularly in the neighbouring Namibia, a similar arid area (Scacchia *et al.* 2009). Limited light trap surveys in Namibia show that, similarly to Namaqualand, *Culicoides* populations are relatively low (Becker *et al.* 2012).

The present study shows that despite the great variation in climatic conditions, *Culicoides* midges and the associated orbiviruses are present in all the 3 South African areas studied: Onderstepoort, Mnisi, and Namaqualand. The study also demonstrates that there are no vector-free periods in these areas. This may suggest that the re-emergence of orbiviruses in early Summer is likely due to over-wintering rather than a reintroduction (Wilson *et al.* 2008).

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