# Occurrence of bluetongue virus in *Culicoides* midges collected at Mnisi, Mpumalanga Province, South Africa.

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## Abstract

Background: Bluetongue (BT) is a non-contagious, vector-transmitted disease caused by the bluetongue virus (BTV) of the *orbivirus* genus in the family *Reoviridae*. BT is a disease mainly of ruminants with sheep being the most susceptible. The vector-borne spread of BTV by adult females of specific *Culicoides* species comprises the most important route of transmission of BT. The occurrence of different *Culicoides* vectors and BTV serotypes varies throughout the world. Throughout the world, sequential identification of BTV is performed. At present, BTV is considered present on all continents except AntarcticaAt present, there are 26 different serotypes of BTV recognised, with the majority being originally of African origin.

Aim of the study: The aim of this project was to identify which *Culicoides* species are present in different areas of Mnisi within the Mpumalanga Province in South-Africa and to determine whether BTV is present in those *Culicoides* midges.

Methods: Biting midges of the genus *Culicoides* were caught using Onderstepoort black light traps. A total of 48 collections were made at 10 different trap sites in Mnisi during 13 sampling nights between June 18 and July 10 2013. The midges were allocated in pools of approximately 200 midges per pool. One pool per trap site were morphologically pre-identified to species, gender and parity level by an entomologist. Midges were homonogised, RNA extraction was performed by adding Trizol reagent to the insect debris. RNA purification of the Trizol reagent extracts was performed by using Qiagen RNeasy Minelute columns according to the protocol of the manufacturer. To determine the presence of BTV, a RT-PCR assay using BTV segment 10 as target was used.

Results: A total of 18 561 midges were captured. After differentiation of 1 614 midges (8.7% of total midges caught), 20 *Culicoides* species were recognized, with *C. imicola* being the dominant species. A total of 48,5% of differentiated midges were either parous (26,6%), bloodfed (0,7%) or gravid (21,2%), with the remaining being nulliparous (35,4%) or male (16%). The qRT-PCR tested positive for BTV segment 10 in 63 out of 82 midge pools. A total of 19 samples did not show amplification of BTV.

Conclusion: The parity rate found in this research are quite high, which serves as an indicator of increased survival rates in a population. Therefore, midges might have been feeding frequently on nearby livestock. The qRT-PCR tested positive for BTV segment 10 in 63 out of 82 midge pools. A total of 19 samples did not show amplification. The infection rate of BT in *Culicoides* midges in this study is estimated to be 0,73. These results show that BTV is present within Mnisi. However, positive qRT-PCR results do not reflect that vector competent *Culicoides* midges are present to transmit BT to other animals since midge species were not tested separately. To address the question whether vector competent *Culicoides* midges are present within Mnisi, virus isolations of individual BTV-positive midges and oral susceptibility studies are indicated.

Keywords: Biting midges, *Culicoides*, polymerase chain reaction, bluetongue, bluetongue virus.

## Theoretical background

Bluetongue (BT) is a non-contagious, vector-transmitted disease caused by the bluetongue virus (BTV) of the *orbivirus* genus in the family *Reoviridae*. BT is a disease mainly of ruminants with sheep being the most susceptible, but also camelid species and some wild ruminants can become infected (Maclachlan *et al*., 2009). At present, there are 26 different serotypes of BTV recognised (Maan *et al*., 2011).

### The virus

BTV is a dsRNA virus that has ten linear genome segments located within the core particle. The genome segments are surrounded by a protein capsid (Mellor and Wittmann, 2002; Breard *et al*., 2004). The genome encodes for seven structural (VP1-VP7) and 5 non-structural proteins (NS1, NS2, NS3, NS3/A, NS4) (van Dijk and Huismans, 1988; Belhouchet *et al*., 2011). The inner core exists of minor proteins VP1, VP4 and VP6, and the major proteins VP3 and VP7 (Breard *et al*., 2004). Genome segment 5, which encodes NS1, is considered to be the most suitable segment to diagnose BTV (Huismans and Cloete, 1987). Bluetongue viruses can be classified according to serotype due to variation in the outer capsid proteins (Huisman and Erasmus, 1981). The outer capsid consists of two virus specific proteins which provide antigenic variability of the virus, namely VP2 and VP5 (Breard *et al*., 2004). VP2 in particular can be used to categorize field BTV according to serotype (Huismans and Cloete, 1987).

When infection of multiple virus serotypes or strains in the same animal or insect is present, reassortment of individual gene segments can take place, resulting in more genetic variety (Maclachlan *et al*., 2009). Through selection for an extended period, genetically distinct virus strains can emerge. As a consequence, this genetic variety leads to differences in phenotypical features of each virus strain, including their virulence. In this way, serotype alone does not determine virulence (Maclachlan *et al*., 2009). For example, BTV of a certain serotype may cause insignificant disease in animals, while another strain of the same serotype causes high mortality rates.

Besides the categorisation based on serotype, BTVs can also be divided according to phylogenetic analysis of nucleotide sequences from each of the genome segments i.e. topotypes. This results into a separation of bluetongue viruses of a western lineage which circulate in Africa, the Caribbean and America, and those of the eastern lineage, which occurs in Asia, Indonesia and Australia (Carpi *et al*., 2010).

Pathogenesis

Some *Culicoides* midge species serve as vectors of BTV. When these *Culicoides* species feed on a viraemic animal, the virus is ingested by the midge. The virus will attach to the luminal surface of the gut and invade gut cells by means of direct penetration or receptor-mediated endocytosis. In the gut cells the virus will replicate after which particles escape through the basolateral side to enter the haemocoel. Many secondary target organs then become infected, including the salivary glands. After replication in the salivary glands, virus is accumulated in the salivary ducts and then released with the saliva of the midge into the host during a subsequent blood meal (Mellor *et al*., 2000). As the virus will replicate in the insects tissues, the individual midge will remain infected throughout its lifetime (Quinn, 2001).

When BTV is transmitted from vector to host it will travel to the regional lymph node of the host and initial replication follows. The virus then is released into other tissues where it invades endothelial cells, mononuclear phagocytic and dendritic cells of the host and cause damage to these cells during virus replication (Quinn, 2001; Breard *et al*., 2004; Maclachlan *et al*., 2009). Clinical manifestations of BT are due to vascular damage, which leads to coagulopathy, tissue infarction, necrosis and excessive bleeding (Maclachlan, 2009). It is shown *in vitro* that necrosis of infected endothelial cells is the cause of increased permeability of the endothelium. In addition, infection of ruminant macrophages leads to production of cytokines that also increase the permeability of endothelium because of alterations in cell cytoskeleton and adherens junctions, without any necrosis (Maclachlan *et al*., 2009; Maclachlan, 2011). Both mechanisms lead to leakage of fluids, with the resultant shock being responsible for high mortality rates. The direct damage to the vascular endothelium is thought to be causative for tissue ischemia and necrosis.

Clinical symptoms

BT mainly affects sheep, but also other ruminants, camelids, deer and many wild African herbivore species can become infected (Mellor *et al*., 2000; Mellor and Wittmann, 2002; Breard *et al*., 2004). Clinical signs associated with BT may vary depending on susceptibility of the host, individual features of the host (i.e. age, breed, nutritional status and immune status), the virulence of the strain involved and environmental factors (Mellor and Wittmann, 2002; Breard *et al*., 2004). Infection with BTV may lead to subclinical disease in cattle and goats within endemic areas, with these host species serving as amplifying hosts of the virus (Maclachlan *et al*., 2009). Also, sheep that are native to areas where BT is endemic usually do not show clinical signs (Maclachlan *et al*., 2009). However, infection with BTV can result in severe clinical illness with a high morbidity and mortality rate in susceptible ruminants, especially certain European breeds of sheep seem very prone to the disease (Gibbs and Greiner, 1994; Maclachlan *et al*., 2009).

The incubation period in sheep is usually 2-9 days after infection and, when they do not succumb to the disease, they remain viraemic for approximately 60 days (Mehlhorn *et al*., 2008). Lesions seen in susceptible sheep include oral erosions and ulcers, lameness and coronitis, weakness, depression, anorexia, fever and facial edema (Mellor and Wittmann, 2002; Breard *et al*., 2004; Mehlhorn *et al*., 2008; Maclachlan *et al*., 2009; Maclachlan, 2011). Death may follow in 8-10 days (Breard *et al*., 2004). Animals showing severe symptoms of BT may either die, develop chronic disease with wasting or recover (Maclachlan *et al*., 2009). Mortality rates in susceptible sheep vary between 2% and 30%, but in some cases can even reach 70% (Breard *et al*., 2004; Coetzee *et al*., 2012).

Cattle and goats from a naïve population can show similar signs, as was the case with the BTV serotype 8 outbreak in Europe in 2006 (Maclachlan *et al*., 2009). However, in endemic areas they are considered to act as amplifying hosts and are sub-clinically affected. It is thought that this is due to a prolonged viraemia that occurs when the disease remains subclinical (Barratt-Boyes and Maclachlan, 1994; Breard *et al*., 2004; Mehlhorn *et al*., 2008).

Also, non-herbivores such as dogs and large African carnivores such as lions and Eurasian lynx can become infected with BT by feeding on infected carcasses and/or foetuses

(Alexander *et al*., 1994; Jauniaux *et al*., 2008). However, the epidemiological contribution of this oral route of infection remains uncertain.

Routes of transmission

The vector-borne spread of BTV by adult *Culicoides* remains by far the most important way of transmission of BT. The virus may also be transmitted by other routes including mechanical, venearal, transplacental and oral transmission (Mellor and Wittmann, 2002; Breard *et al*., 2004; Menzies *et al*, 2008; Maclachlan *et al*., 2009). When pregnant sheep or cattle become infected with certain strains of BTV, e.g. BTV-8, transplacental infection of the foetus will result in either still births, abortions or the birth of neonates with severe central nervous system abnormalities which are not compatible with life (Maclachlan *et al*., 2009). It is thought that these forms of transmission only occur occasionally in wildtype strains, with the exception of BTV-8 . However, the exact role of these routes of transmission (i.e. mechanical, venereal, transplacental and oral transmission) of BTV still needs to be clarified.

*Culicoides* vector

*Culicoides* midges are small biting flies belonging to the family *Ceratopogonidae* and serve as vectors for BTV (Figure 1.). Female *Culicoides* feed on blood of mammals and birds whereas males do not need to feed on blood. Females lay their eggs in various kinds of habitats depending on species, ranging from pools and streams to rotting fruit and animal dung. After hatching, *Culicoides* larvae go through four stages of development. The pupal stage is brief and after about two to three days adult *Culicoides* emerge. In general, most adult midges are active between dusk and dawn, with peak activity levels around sunset and sunrise. Midges typically have a short lifespan of 10-20 days, although some individuals may survive for much longer periods of 44-90 days under favourable conditions. During their lifetime, females consume multiple blood meals (Mellor *et al*., 2000).

**Figure 1.** Female *Culicoides zuluensis* (Meiswinkel *et al.*, 2004)

*Culicoides* midges can fly a maximum of 2 km, however wind movements influence the long-range distribution of the midges. Suitable winds on which midges can travel will be at heights of 0.5 to 2 km, wind velocities of 10 to 40 km/h and temperatures of 12.8°C to 35.8°C (Sellers, 1992; Mellor *et al*., 2000). In a single night the wind can carry the midges up to hundred kilometers. It has been considered that it might even be possible that midges are blown by the wind for 700 km (Sellers, 1992; Mellor *et al*., 2000; Purse *et al*., 2005). Therefore, it is highly likely that BT can be introduced by means of wind-borne dispersal of infected midges.

Over 1400 species of *Culicoides* are currently identified of which only approximately 30 species have been demonstrated to act as a vector of BTV (Meiswinkel *et al*., 2007; Mellor *et al*., 2000). Differentiation of *Culicoides* to species level relies on distinct wing patterns and requires a thorough knowledge of morphology and experience with the identification of midges. Especially differences between wing patterns of female members of a single species complex are very subtle which severely hampers morphological differentiation (Lehmann *et al*., 2012). Various *Culicoides* species are found in different regions of the world, although in some areas, such as Asia, more research should be conducted to investigate which species are present.

The distribution of BTV is strongly related to the presence of vector competent *Culicoides* species that are able to transmit the disease, as well as environmental conditions that support large numbers of these vectors (Mellor *et al*., 2000; Mellor and Wittmann, 2002; Maclachlan, 2011). High activity rates, low rates of dispersal, rapid larval development, low adult mortality and breeding throughout the entire year are variables that will favour high abundance at a particular site. In a similar manner, vector capacity (e.g. the ability of a vector population to transmit the virus to a host) is determined by various factors such as vector competence (e.g. the capacity for the virus to develop in the vector), vector abundance, biting rates, host preferences, survival rates and duration of development of virus in the vector (Mellor and Wittmann, 2002; Venter *et al*., 2010). Variation in environmental conditions, in particular high temperatures, exert influence on all these factors (Mellor *et al*., 2000; Mellor and Wittmann, 2002).

Global distribution of *Culicoides* vector

The occurrence of different *Culicoides* vectors and BTV serotypes varies throughout the world. Specific *Culicoides* species that act as vectors are associated with different BTV serotypes and/or strains in relatively distinct global ecosystems (Maclachlan, 2011).

Africa *Culicoides imicola* is the major vector competent species of the African continent (Mellor *et al*., 2000; Tabachnick, 2004). However, cooler and more arid areas of southern Africa also experience BT but *C. imicola* is absent in those areas. Here *C. bolitinos* is considered to be the major vector of BTV (Mellor *et al*., 2000; Tabachnick, 2004) *C. bolitinos* is a sibling species of *C. imicola* and breeds in the dung of cattle. Besides these two *Culicoides* species, BTV has also been isolated from other species, such as *C. tororoensis* and *C. milnei* in Kenya. However, their role in BT epidemiology is still uncertain (Mellor *et al*., 2000).

Asia *C. imicola* is identified in many countries of the Asian continent, however only limited research on the occurrence of different *Culicoides* species has been performed. More studies on vector distribution and abundance, virus isolation studies, vector competence studies and oral susceptibility studies are required. Only in Indonesia studies have been published

showing the existence of almost 50 *Culicoides* species, including four vector competent species (*C. atoni, C. brevitarsis, C. fulvus* and *C. wadai*) (Mellor *et al*., 2000).

Australia *C. fulvus, C. wadai, C. actoni* and *C. brevitarsis* are suggested to be the most important vectors of BTV in Australia, although other *Culicoides* species are thought to also be able to transmit BTV (Mellor *et al*., 2000; Tabachnick, 2004). *C. fulvus* is considered the most efficient but is only able to survive in regions with high summer rainfall and therefore is not abundant in drier areas where most sheep are present. It is believed that *C. wadai* has crossed the sea from Indonesia into Australia (Mellor *et al*., 2000).

America Historically it was presumed that *C. variipennis* was the major vector throughout North America. However, recent research has shown that *C. variipennis* is in fact a complex, consisting of subspecies *C. v. variipennis,C. v. occidentalis* and *C. v. sonorensis* (Mellor *et al*., 2000). It is thought that occurrence of BTV serotypes 2, 10, 11, 13 and 17 is dependent upon the presence of *Culicoides v. sonorensis*, which is now believed to be the major vector of BTV in America (Mellor *et al*., 2000; Tabachnick, 2004). Field isolation studies, vector competence studies and oral susceptibility studies show that *C. v. variipennis* is far more resistant to oral infection then *C. v. sonorensis* (Mellor *et al*., 2000). In addition, in the northeast of America where *C. v. variipennis* is considered to be the only species of the complex present, BT has not been identified (Mellor *et al*., 2000). In south-eastern United States BTV serotype 2, 3 and 6 are associated with the distribution of *Culicoides insignis*. In this region, BTV 3 and 4 are also isolated from *C. pusillus* (Mellor *et al*., 2000; Maclachlan, 2011).
In Central America and the northern part of South America, it is believed that *C. insigni* is the major vector competent *Culicoides* species and is thought to be associated with BTV serotypes 1, 3, 6, 8 , 12 and 14 (Tabachnick, 2004).

Europe *Culicoides imicola* was considered the traditional African-Asian vector of BT present in countries surrounding the African continent such as Spain, Portugal, Greece, Cyprus, Israel, Turkey, Yemen and Oman. These countries all showed serological or clinical evidence of BT at some stage (Mellor *et al*., 2000; Mellor and Wittmann, 2002). However, the spread of BTV into northern Europe where *C. imicola* was not found in any of many *Culicoides* surveys, suggested that other species are involved (Maclachlan, 2012; Meiswinkel *et al*.,

2007, 2008). *Culicoides obsoletus sensu strictu*, *Culicoides pulicaris, Culicoides dewulfi, Culicoides scoticus* and *Culicoides chiopterus* were known to be present in Europe long before BT spread into the region, suggesting that changes in climate might serve as a factor in the ability of these vectors to transmit BTV (Maclachlan, 2011; Meiswinkel *et al*., 2007, 2008). Recent studies presume that the *Obsoletus* complex (that consists of the three species *C. obsoletus, C. scoticus, C. chiopterus*), *C. dewulfi*, *C. pulicaris* and *C. achrayi* are vector competent species responsible for the transmission of BTV in Europe (Meiswinkel *et al*., 2007, 2008; Mehlhorn *et al*., 2008; Lehmann *et al*., 2012; Del Rio *et al*., 2013). Members of the *Obsoletus* complex are considered the most important vector due to their widespread occurrence in Europe and high survival rates (Mellor and Wittmann, 2002; Meiswinkel *et al*., 2008).

South Africa  It is believed that at least thirteen *Culicoides* species may play a significant part in the epidemiology of BT in South Africa. The *Culicoides* species involved are *C. imicola, C. bolitinos, C. gulbenkiani, C. zuluensis, C. milnei, C. huambensis, C. expectator, C. magnus, C. enderleni C. leucostictus, C. pycnostictus, C. bedfordi* and *C. angolensis* (Venter *et al*., 2010). BTV has been isolated from many *Culicoides species*, including *C. imicola, C. bolitinos, C. milnei, C. pycnostictus* and *C. expectator,* which are South African species associated with livestock (Venter *et al*., 2010). Only *C. imicola* and *C. bolitinos* are considered significant vectors of BT in South Africa (Venter *et al*., 2010; Coetzee *et al*., 2012). *C. imicola* serves as the dominant vector of BT and breeds predominantly in wet soils enriched with dung (Mellor and Wittmann, 2002). The relatively low vector competence of *C. imicola* is probably compensated for by massive numbers of these midges around livestock (Venter *et al*., 2010). *C. bolitinos* is most dominant in the colder regions of South Africa and breeds mainly in the dung of herbivores (Nevill *et al*., 1988; Paweska *et al*., 2002). Research performed by Venter *et al*. (2010) shows that *C. bolitinos* has a higher oral susceptibility for BTV and is able to replicate the virus to higher titres as compared to *C. imicola*. In addition, *C. bolitinos* may also be able to replicate the virus at lower temperatures then *C. imicola* (Paweska *et al*., 2002).

A very important factor in the epidemiology of BT is whether vector competent species of *Culicoides* persist throughout the year or are absent when conditions do not favour their

survival. In tropical regions BTV is present throughout the entire year, whereas in areas with a temperate climate the occurrence of BT follows a certain seasonal distribution, with the majority of infections occurring during late summer and autumn months (Maclachlan *et al*., 2009). If adult midges disappear for a certain period and no amplifying hosts are present, introduction of BT is dependent on the entrance of the virus from elsewhere. Mechanisms of introduction in those areas could be by infected *Culicoides* carried by the wind or by introduction of infected hosts (Mellor *et al*., 2000). Annual absence of adult midges for a certain period could be explained by environmental conditions that do not favour breeding (i.e. seasonal rainfall, wind speeds and temperature) (Mellor *et al*., 2000).

Global distribution of BTV

Sequential identification of BTV is performed throughout the world. These studies led to the belief that BTV is emerging from its presumed ancestral origin in Africa (Gibbs and Greiner, 1994; Mehlhorn *et al*., 2008). At present, BTV is considered present on all continents except Antarctica. Currently the disease is identified between latitudes of 40º North and 35º South, except specific regions in Asia and North America where BT is present as far as 50º North. Also, northern Europe is affected (Mellor *et al*., 2000; Mellor and Wittmann, 2002; Breard *et al*., 2004; Tabachnick, 2004). A schematic overview of the worldwide distribution of BTV is shown in Figure 2.

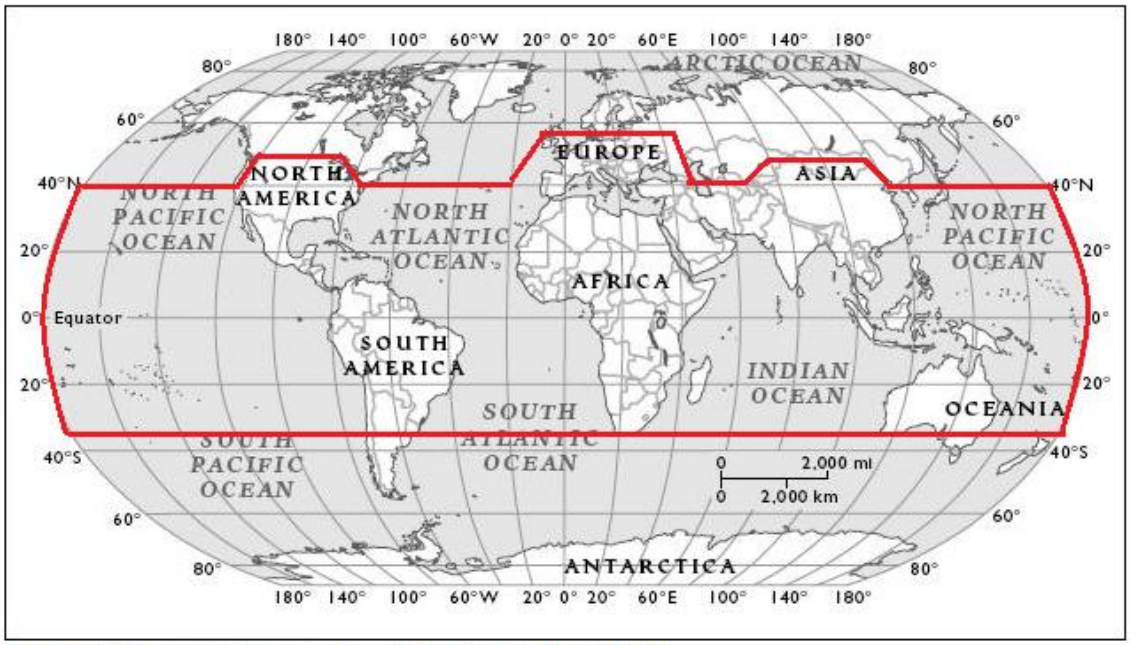


**Figure 2.** A schematic overview of the distribution of BTV

The first report of BT was made in late 19th century when susceptible European Merino sheep were imported in South Africa, although it has been proposed that BT has probably been endemic in wild ruminants without any clinical signs before that time (Breard *et al*., 2004; Verwoerd, 2012). Around two decades later the disease was identified in western Africa and several other countries in sub-Saharan Africa (Breard *et al*., 2004). BT was first detected outside Africa in North America in 1940 and Cyprus in 1943 (Breard *et al*., 2004).

Europe The occurrence of BTV initially remained relatively infrequent in Europe. Several outbreaks occurred in countries around the Mediterranean Sea (Spain and Portugal, 1956-1960, BTV-10) (Greek Islands, 1979-1980, BTV-4), although they were not able to persist (Mellor and Wittmann, 2002; Breard *et al*., 2004; Wilson and Mellor, 2008). However, since 1998 changes in distribution of BTV were detected. BTV serotypes 1, 2, 4, 6, 8, 9, 11 and 16 have entered Europe since 1998 (Wilson and Mellor, 2008; Maclachlan, 2011). Wilson and Mellor (2004) state that these serotypes invaded Europe by means of three routes (shown in Figure 3) and at least one BTV serotype has been active in southern Europe every year since 1998 (Wilson and Mellor, 2004). It is believed that all serotypes currently identified in the Mediterranean area after 1998 are of either a western lineage or eastern lineage. Western strains have emerged from the African continent, whereas eastern strains have entered Europe from Asia (Purse *et al*., 2005; Maclachlan, 2011).


**Figure 3.** Three principal routes of BTV introduction into Europe (Wilson and Mellor, 2008)

Serotype 8 was first discovered during the 2006-2008 outbreak of BT in northern Europe (Mehlhorn *et al*., 2008). This particular serotype had never been identified in other parts of Europe before. It was found to be closely related phylogenetically to a 1982 sub-Saharan West African strain, suggesting that BT may have been translocated over a long distance (Meiswinkel *et al*., 2007; Carpenter, 2011). However, there is no certainty about the origins of BTV-8 and how it was introduced into Europe. It is most likely that the virus was introduced through the importation of viraemic ruminants from endemic areas or through windborne transportation of infected midges over a long distance (Mehlhorn et al., 2008; Carpenter, 2011). However, other routes of introduction are also possible, although less likely, such as introduction of non-ruminant viraemic animals, movement of infected female midges through shipments of animals or plants and contaminated biological products (i.e. vaccines or cell cultures) (Carpenter, 2011).

A remarkable feature of the European BTV-8 strain is that it is highly virulent, not only for sheep but also cattle and wild ruminants. This serotype also crossed the placental barrier to infect the foetus very frequently, which is very unusual considering that this is only a rare event with other serotypes (Desmecht *et al*., 2008). This specific serotype has now spread throughout the Mediterranean area, to above latitudes of 50º North and also managed to enter certain areas of Scandinavia (Agren *et al*., 2010).

North America Serotypes 1-3, 5, 6, 9-14, 17, 19, 22 and 24 have been demonstrated to be present in the south eastern United States. The assumption is that most of these serotypes spread into North America from the nearby Caribbean area where they are believed to be enzootic (Maclachlan, 2011).

South Africa Most sheep in South Africa are domestic breeds which are not naïve and therefore do not show clinical signs associated with BT. Most outbreaks of BT occur in the population of susceptible European breeds which is present (Gibbs and Greiner, 1994; Maclachlan *et al*., 2009). BT in South Africa is most common between February and April during late summer and autumn, in particular when there is a lot of rainfall. In regions with a colder climate, the province of the Free State for example, the disease is not revealed following early winter frosts. In contrast, it is presumable that in the warmer regions at the

east side of the country, the disease can be transmitted year round (Coetzee *et al*., 2012). Based on a limited number of published field surveys it is stated that 22 of 26 serotypes are present in South Africa, serotypes 20, 21, 25 and 26 are thought to be exotic. About 14 to 18 different serotypes circulate every season, although the occurrence of specific serotypes changes yearly. Usually three to five dominant serotypes are present, which are then replaced by other dominant serotypes the following season, only to become dominant again a few years later. These changes in the predominance of serotypes are most likely attributable to herd immunity (Coetzee *et al*., 2012).

Other parts of the world  To the east of Europe, in Anatolian Turkey, Syria and Jordan, BTV serotypes 2, 4, 6, 9, 10, 13 and 16 have been identified in the past, of which some have spread westward into Europe. Previously, only BTV serotypes 2, 4, 6, 10 and 16 were present in Israel, but since then serotypes 8, 15 and 24 have also been introduced in the country (Maclachlan, 2011). Serotypes 1, 3, 9, 15, 16, 20, 21 and 23 were historically present in Australia. However, recently serotypes 2 and 7 have been detected in northern Australia in 2007/2008 (Maclachlan, 2011). Because of the lack of sufficient monitoring for BTV in other parts of the world it is complicated to interpret the BTV status in those areas.

It is suggested that the emergence of BTV into northern regions which were thought to have unsuitable climate conditions to sustain BT, may be a result of global climate change (Mellor and Wittmann, 2002; Meiswinkel *et al*. 2007, 2008; Maclachlan, 2011; Del Rio *et al*., 2013).

## Aim of study

The aim of this project was to determine whether BTV is present in *Culicoides* midges in different areas of Mnisi, Mpumalanga Province in South-Africa.

The aim of this project can be divided into the following objectives:

- To determine which species of *Culicoides* are prevalent in the assigned areas
- To investigate whether *Culicoides* midges present in the assigned areas are infected with BTV

## Expected outcome

Based on previous research conducted by Venter *et al*. (1998), it is expected that *Culicoides imicola* would be the most prevalent midge species found in Mnisi. Also, BTV infected midges will be of low prevalence.

## Material and methods

### Research area

Mnisi is situated in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province in South-Africa (Figure 4.). Within this area, cattle, goats, chickens and donkeys are the main livestock species present, but also game parks with wildlife are present. A frail fence forms the only barrier between livestock and wildlife and contact between the two is possible (University of Pretoria, n.d.).

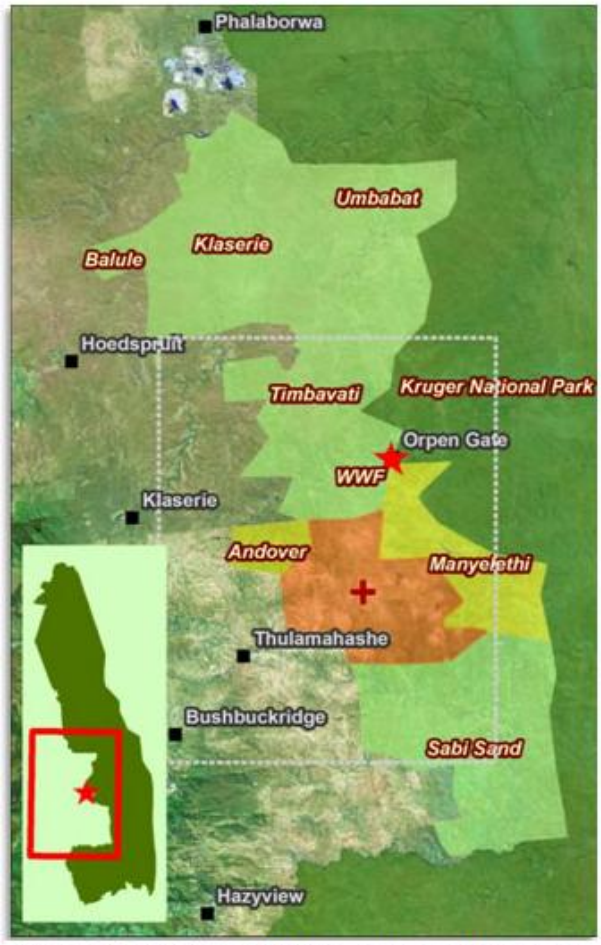


**Figure 4.** The area of Mnisi - indicated in the red coloured area. Reprinted from University of Pretoria, http:/web.up.ac.za/default.asp?ipkCategoryID=17694&sub=1&parentid=16376&subid=16645&ipklookid=13

In order to address the objectives of this study, five sites were chosen within Mnisi to collect midges. These sites were located within the villages of Hluvukani, Athol, Hlalakahle, Ludlow and Welverdiend. These particular sites were chosen because of the lack of information on midge species abundance and the high number of domestic and wild ruminants that are present. Also, the occurrence of BTV in Mnisi is of importance as neighbouring farms are present that contain livestock and because of the close proximity of the Kruger National Park. Domestic ruminants live in close proximity to wild ruminants. As such, Mnisi serves as a suitable site to investigate the occurrence of BTV at the domestic-wildlife interface.

Collection and storage of *Culicoides* midges

Four 220 V Onderstepoort light traps equipped with 30 cm 8 W black light tubes were used to collect live midges through a suction fan into 500 ml plastic cups filled with 5% Savlon antiseptic solution (Venter *et al*., 2009). The Savlon solution decreases the surface tension so that when midges that attempt to sit on the water surface will sink into the solution. The Savlon solution also has an antiseptic function to allow the midges to be stored until further use. Traps were placed as close to cattle as possible where high densities of *Culicoides* midges are present (Garcia-Saenz *et al*., 2011; Venter *et al*., 2012). Mosquito netting placed around the trap prevented large insects from entering the trap. Traps were installed in June and July of 2013 between dusk and dawn (Appendix A.1 and A.2). At collection, the cups containing midges were put in a small container with ice packs in order to keep the midges cooled during transport. At the laboratory the midges were washed two times using PBS+ solution containing 500 IU penicillin, 500 µg/ml streptomycin, 500 µg/ml gentamycin and 1.25 µg/ml amphoterecin B to clear the external surface of the insects from contamination. After marking the specimen jars according to trap site, the midges were stored in PBS+ solution in a fridge at 4°C. Orbiviruses like BTV are double stranded RNA viruses and their nucleic acids will be preserved in Savlon or cooling at 4 °C for several weeks.

Determination of *Culicoides* species

Field caught midges from each sample were separated from other insects and pooled using approximately 200 midges per pool. The tubes were marked according to the collection site. One pooled sample from each collection site was used to differentiate *Culicoides* according to species, gender and parity using Dyce's (1969) method by an independent entomologist.

Processing of *Culicoides* midges

After pooling, the midges were washed in PBS+ and 1 ml reconstituted Eagle's minimum essential medium MEM (without serum) was added. The MEM-solution contains 500 IU penicillin, 500 µg streptomycin, 500 µg gentamycin and 1.25 µg amphoterecin B per ml and sterile FCS 5%. After adding a sterile glass bead the midges were homogenised using the TissueLyser at full speed for two minutes. All of the remaining MEM was separated from the insect debris. RNA extraction was performed by adding Trizol reagent to the MEM according to the protocol of the manufacturer (Appendix B.1). Double stranded RNA purification of the Trizol reagent extracts was performed by using Qiagen RNeasy Minelute columns according to the protocol of the manufacturer (Appendix B.2). The resultant total RNA products were analysed using a Spectral Photometer to obtain an indication of concentration and purity. The RNA products were denatured by adding 10% DMSO and heating the samples at 95°C for 3 minutes, after which the denatured RNA was snap cooled on ice (-20°C) for 5 minutes. A one step real-time qRT-PCR targeting segment 10 of the BTV genome with NS3 primers and a sequence specific probe was performed by using TaqMan® Fast Virus Master Mix reagents (Applied Biosystems) and an Applied Biosystems StepOnePlus PCR machine. Ten pmol concentrations of the forward primer (5'-GTGTCGCTGCCATGCTATC-3') and reverse primer (5'-GTACGATGCGAATGCA -3') were used. Each reaction contained 5 µl master mix, 1 µl forward primer, 1 µl reverse primer, 2.5 pmol TaqMan probe (5'-FAM CGAACCTTTGGATCAGCCCGGA MGB-3'), 7 µl of DEPC water and 5 µl RNA. Cycling conditions were as follows; 1 cycle at 50°C for 5 minutes, 1 cycle at 95°C for 20 seconds and 40 cycles at 95°C for 2 seconds followed by 60°C for 30 seconds. Samples were considered positive if they demonstrated a cycle threshold value of <38.

## Results

### Determination of *Culicoides* species

A total of 18 561 midges were captured during 48 collections made between June 18 and July 10 2013. After differentiation of 1 614 midges (8.7% of total midges caught), 20 species were recognized; *C. bedfordi, C. bolitinios, C. brucei, C. coarctatus, C. dekeyseri, C. enderleini, C. expectator, C. glabripernis, C. imicola, C. leucostictus, C. neavei, C. neville, C. nivosus, C. pycnostictus, C. similis, species 54 dark form, C. subschultzei, C. tropicalis, C tutttifruitti* and *C. zuluensis.* An overview of recognised *Culicoides* species per trap site is given in Appendix D.1. A summary of the total number of midges caught per trap site and number of midges differentiated to species level is given in Table 1. An overview of the percentages of each species per trap site of the total number of midges is provided. In addition, the average percentage of each species across all trap sites is presented (see 'mean species %'. For example, *C. imicola* accounts for 50,95% of all 1 614 midges differentiated).

### Determination of *Culicoides* gender and parity

Field caught midges were sorted to species level and gender and parity was determined. Female midges were allocated in one of following categories; nulliparous (no abdominal pigmentation), parous (pigmentation present), bloodfed and gravid. The number and percentage of each gender and/or parity of the 1 614 midges that are differentiated is displayed in Table 2. An overview of parity according to species per trap site is given in Appendix D.1. Female midges (1 355 midges; 83.9%) were present in the captures in larger quantity then males (259 midges; 16,0%), which is a typical result when using light traps, as is indicated by Venter *et al*. (2012).

Table 1. Total number of midges and percentage of each *Culicoides* species according to trap site

| Trapsite | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total number of midges per trap site | 388 | 336 | 610 | 176 | 14.916 | 1.683 | 109 | 120 | 152 | 71 | 18 561 |
| Number of midges differentiated according to species | 200 | 198 | 193 | 176 | 196 | 199 | 109 | 120 | 152 | 71 | 1 614 |
| | | | | | | | | | | | |
| Species % per trap site | | | | | | | | | | | Mean species % |
| *C. bedfordi* | 0,5 | | | 0,6 | 3,1 | 5,0 | 1,8 | 0,8 | 1,3 | | 1,31 |
| *C. bolitinios* | 7,0 | 5,0 | 4,1 | 2,8 | 1,5 | 1,5 | 12,8 | 9,2 | 3,3 | 4,2 | 5,14 |
| *C. brucei* | | | | | 0,5 | 2,5 | | | | | 0,30 |
| *C. coarctatus* | | | | | 0,5 | 2,5 | | | 2,0 | | 0,50 |
| *C. dekeyseri* | | | | | | 0,5 | | | | | 0,05 |
| *C. enderleini* | 1,0 | 1,0 | 1,0 | 0,6 | 1,0 | | 1,8 | 2,5 | 0,7 | | 0,96 |
| *C. expectator* | 1,0 | 13,6 | 5,2 | 1,1 | 0,5 | 1,5 | 2,8 | 5,0 | 0,7 | 5,6 | 3,70 |
| *C. glabripernis* | | | | | | | 0,9 | | | | 0,09 |
| *C. imicola* | 72,0 | 30,3 | 39,9 | 31,3 | 80,6 | 66,3 | 35,8 | 40,8 | 53,3 | 59,2 | 50,95 |
| *C. leucostictus* | 5,5 | 18,2 | 14,5 | 24,4 | 1,0 | 2,0 | 6,4 | 8,3 | 18,4 | 11,3 | 11,00 |
| *C. neavei* | | 0,5 | | 0,6 | 1,0 | 5,0 | | | 0,7 | | 0,78 |
| *C. neville* | 0,5 | | 16,0 | | 3,6 | 3,0 | | | | | 2,31 |
| *C. nivosus* | 1,0 | 2,5 | 3,6 | 7,4 | | 0,5 | 2,8 | 0,8 | 3,3 | 2,8 | 2,47 |
| *C. pycnostictus* | 4,5 | 1,5 | 1,6 | 2,8 | | 1,0 | 3,7 | 2,5 | 2,6 | | 2,02 |
| *C. similis* | 2,0 | 6,6 | 2,1 | 1,7 | 1,0 | 2,0 | 1,8 | 5,0 | 3,3 | | 2,55 |
| *Species 54 DF* | 0,5 | 0,5 | | 1,7 | | 0,5 | 9,5 | 7,5 | | 2,8 | 2,30 |
| *C. subschultzei* | 2,0 | 3,5 | 4,1 | 3,4 | 1,0 | 2,0 | | | 3,3 | 1,4 | 2,07 |
| *C. tropicalis* | 2,0 | 12,6 | 8,3 | 2,8 | 1,0 | 1,5 | 15,6 | 14,2 | 1,3 | 1,4 | 6,07 |
| *C. tuttifrutti* | 0,5 | 3,5 | 14,0 | 18,8 | 1,0 | 2,0 | 3,7 | 3,3 | 5,9 | 11,3 | 6,40 |
| *C. zuluensis* | | 0,5 | | | 0,5 | 0,5 | 0,9 | | | | 0,24 |
| | | | | | | | | | | Total: | 101,21* |
| | | | | | | | | | | | * due to rounding differences does not exactly sum up to 100% |

Table 2.
Number and percentage of midges differentiated according to gender and parity

| | | |
|---|---|---|
| Total number of midges | 18 561 | |
| Number of midges differentiated according to gender and parity | 1 614 | |
| | | |
| Gender and Parity | | |
| Female: Nulliparous | 571 | 35,4% |
| Parous | 430 | 26,6% |
| Bloodfed | 12 | 0,7% |
| Gravid | 342 | 21,2% |
| Male | 259 | 16,0% |

Realtime qRT-PCR results

A BTV bloodfed midge served as a positive control. The positive control displayed amplification of BTV segment 10. No amplification was seen with the negative control. Samples 1.1, 1.4, 1.5, 3.1, 5.1, 5.2, 5.5, 5.7, 5.10, 5.24, 5.26, 5.43, 5.48, 6.1, 6.3-6.5, 6.8 and 8.1 did not show any amplification, while the remaining samples tested positive for BTV segment 10. The results are summarized in Table 3. The associated amplification plot of the test run is displayed in Figure 5.

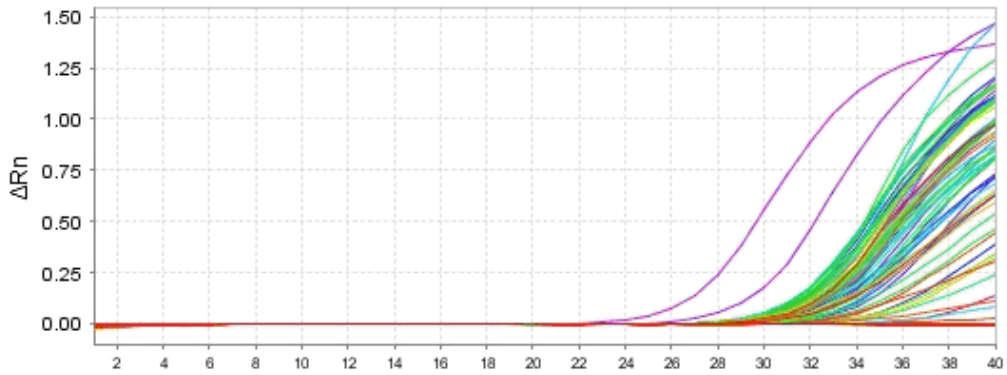


**Figure 5.** Amplification plot. Each colour represents a different sample.

Table 3. RT-PCR results per trap site

| Trap site | CT-value | Result qRT-PCR |
|---|---|---|
| 1.1 | - | No amplification |
| 1.2 | 30,59 | Positive |
| 1.3 | 29,74 | Positive |
| 1.4 | - | No amplification |
| 1.5 | - | No amplification |
| 2.2 | 32,47 | Positive |
| 3.1 | - | No amplification |
| 3.2 | 38,48 | Negative |
| 3.3 | 32,69 | Positive |
| 4.1 | 34,24 | Positive |
| 5.1 | - | No amplification |
| 5.2 | - | No amplification |
| 5.3 | 29,75 | Positive |
| 5.4 | 33,35 | Positive |
| 5.5 | - | No amplification |
| 5.6 | 30,94 | Positive |
| 5.7 | - | No amplification |
| 5.8 | 29,74 | Positive |
| 5.9 | 29,17 | Positive |
| 5.10 | - | No amplification |
| 5.11 | 31.24 | Positive |
| 5.12 | 29.28 | Positive |
| 5.13 | 29,84 | Positive |
| 5.14 | 28,83 | Positive |
| 5.15 | 29,18 | Positive |
| 5.16 | 28,99 | Positive |
| 5.17 | 31,93 | Positive |
| 5.18 | 29,38 | Positive |
| 5.19 | 31,49 | Positive |
| 5.20 | 29,95 | Positive |
| 5.21 | 28,78 | Positive |
| 5.22 | 29,07 | Positive |
| 5.23 | 30,45 | Positive |
| 5.24 | - | No amplification |
| 5.25 | 33,72 | Positive |
| 5.26 | - | No amplification |
| 5.27 | 30,87 | Positive |
| 5.28 | 29,64 | Positive |
| 5.29 | 35,97 | Positive |
| 5.30 | 29,9 | Positive |
| 5.31 | 30 | Positive |
| 5.32 | 29,25 | Positive |
| 5.33 | 28,69 | Positive |

| Trap site | CT-value | Result qRT-PCR |
|---|---|---|
| 5.34 | 29,64 | Positive |
| 5.35 | 29,88 | Positive |
| 5.36 | 29,95 | Positive |
| 5.37 | 31,51 | Positive |
| 5.38 | 30,13 | Positive |
| 5.39 | 29,43 | Positive |
| 5.42 | 30,45 | Positive |
| 5.43 | - | No amplification |
| 5.44 | 31.55 | Positive |
| 5.45 | 29,11 | Positive |
| 5.46 | 30,43 | Positive |
| 5.47 | 33,35 | Positive |
| 5.48 | - | No amplification |
| 5.49 | 30,91 | Positive |
| 5.50 | 29,95 | Positive |
| 5.51 | 29,08 | Positive |
| 5.52 | 29,09 | Positive |
| 5.53 | 30,98 | Positive |
| 5.54 | 30,4 | Positive |
| 5.55 | 31,89 | Positive |
| 5.56 | 30,78 | Positive |
| 5.57 | 29,81 | Positive |
| 5.58 | 29,17 | Positive |
| 5.59 | 29,73 | Positive |
| 5.60 | 29,85 | Positive |
| 5.61 | 29,51 | Positive |
| 5.62 | 26,71 | Positive |
| 5.63 | 30,19 | Positive |
| 6.1 | - | No amplification |
| 6.2 | 30,2 | Positive |
| 6.3 | - | No amplification |
| 6.4 | - | No amplification |
| 6.5 | - | No amplification |
| 6.6 | 30,35 | Positive |
| 6.8 | - | No amplification |
| 7.1 | - | No amplification |
| 8.1 | - | No amplification |
| 9.1 | 33,74 | Positive |
| 10.1 | 29,76 | Positive |
| Negative | - | No amplification |
| Positive | 24,13 | Positive |

## Discussion

### The use of light traps to obtain a sample of the total midge population

Midge collection by means of light traps is considered the standard and most practical way to monitor and capture *Culicoides* species midges in the field. The Onderstepoort trap is a light and down-draught trap that is equipped with a black light tube and a fan. The Onderstepoort trap collects significantly more midges when compared to other light and down-draught traps such as the Pirbright or Rieb trap (del Rio *et al*., 2013; Venter *et al*., 2009). The 'range of attraction' (e.g. the maximum distance at which insects are attracted to a specific trap) has been studied by many researchers but outcomes differ considerably. In the study by Kirkeby *et al*. (2013), it was found that the range of attraction for *Culicoides* to a CDC 1212 mini light trap was approximately 15,25 metres. Rigot and Gilbert (2012) used Onderstepoort light traps to examine the range of attraction for *Culicoides* and they found it to be approximately 30 meters when traps were running for 30 minute intervals. In contrast, Venter *et al*. (2012) found a range of attraction of 2-4 meters for an Onderstepoort light trap. These differences could be partially explained by variable external factors. The activity rates of adult midges, and thus trapping efficiency, can differ with subsequent nights because of subtle changes in meteorological conditions. These include variables such as wind, temperature and background illumination that can differ due to cloudiness, moon phase and time of sampling related to sunset (Kirkeby *et al*., 2013; Luhken and Kiel, 2012). Light traps and down-draught traps, such as the one used in this study, become less efficient when other sources of light are present and wind speeds increase. Activity is stimulated by decreasing light intensity during sunset and declines thereafter with complete darkness. Activity rates are decreased with increasing wind speeds and activity is at an optimum within a certain temperature range, with specific activity varying between different species of *Culicoides* (Mellor *et al*., 2000). Due to these variables it is difficult to compare results from different collection sites. Further more, it is estimated by Meiswinkel *et al*. (2004) that the Onderstepoort trap is able to collect 1% of the total midge population. In addition, it is a possibility that some *Culicoides* species are more attracted to black light then other species, thus the range of attraction may differ between certain midge species (Venter *et al*., 2012; Kirkeby *et al*., 2013). Due to various reasons mentioned above, it is possible that the midges

caught for the purpose of this research might not be a representation of the whole midge population present in Mnisi but only represent a fraction of the entire population.

Seasonality

In more tropical areas, the annual cycle of *C. imicola* is largely dependent on the rainy season. A temperature related drop in adult population size does not occur in those areas when winter temperature remains high enough. In areas with relativity cold winters such as South Africa, seasonality of *C. imicola* (and probably also other species) is affected by temperature (Mellor *et al*., 2000). The collection of midges with regard to this study was performed during winter time when temperatures are much lower then in summer. This most likely led to a lower number of midges caught and possibly other species present when compared to summer months.

Number of midges caught

The number of midges caught per trap site differs widely. As is shown in Table 1, a total of 14916 midges were captured at trap site 5, which would make up to 80.4% of the total number of midges caught. This difference cannot be fully explained by the fact that midges were caught during more sampling nights at trap site 5. At site 1, 2, 5 and 6 midges were caught during six nights in total. At site 3, 4, 7 and 8, light traps were present for only five nights and only two nights at site 9 and 10. This variation is due to logistical reasons. Since the distance of the trap to the animals is comparable with the other trap sites, that is probably not an explanation for the great number of midges caught at trap site 5. The dominant species found at trap site 5 is *C. imicola* (80,6%), which breeds predominantly in wet soils enriched with dung (Mellor and Wittmann, 2002). Since a water source (i.e. lake) was present near trap site 5, it might be possible that the area around trap site 5 served for breeding purposes. When it comes to mosquitoes, Service (1977) reported that the number of mosquitoes caught using a light trap is significantly increased when the trap is placed near the flight paths of females and near aggregation sites of suitable hosts. Therefore, the quantity and quality of captured *Culicoides* by means of a light trap is not only dependent on weather conditions but also on trap location. This may indicate that the trap at site 5 met these indicators (i.e. trap location near breeding area of *Culicoides* and flight paths to suitable hosts) and hence the great number of midges caught at that particular site.

Determination of *Culicoides* species and parity

Even though a highly experienced entomologist is able to differentiate midges to species level, it might be possible that some midges are classified incorrectly. Recently, Lehmann *et al*. (2012) showed that a multiplex PCR is a reliable diagnostic tool for the differentiation of members of the *Obsoletus* group. Therefore, molecular methods could aid in the identification of *Culicoides* species in future studies. Although misidentification might have happened in a very small portion of the midges differentiated in the current study, it is obvious that *C. imicola*, which accounts for 50.95%, is the dominant species found in this study. This is in accordance with previous studies performed by Venter *et al*. (1998, 2010).

Male midges do not feed on animals so they are not able to transmit BTV. Nulliparous female midges have not previously had a bloodmeal, which leaves only parous, bloodfed and gravid midges being able to transmit BTV. Since it is very time consuming to identify all individual midges according to species and parity, only one pool of every trap site was differentiated. Therefore it was not possible to test only female *Culicoides* which are able to transmit BT in the qRT-PCR, but also nulliparous female midges and male midges were present in pooled samples.

Meiswinkel *et al*. (2008) and Mellor and Wittman (2002) stated that a high parity rate (e.g. the proportion of parous females per collection) serves as an indicator of increased survival rates in a population and therefore midges might have been feeding frequently on nearby livestock. It is quite likely that this enhances the vector capacity of competent midges. Parous, bloodfed and gravid midges comprises the part of the population that might be able to transmit BTV since they fed on animals before. In the current study, 48,5% of all midges are either parous (26,6%), bloodfed (0,7%) or gravid (21,2%), with the remaining being nulliparous (35,4%) or male (16%). Thus, the proportion of midges that might be able to transmit BTV when it comes to gender and the parity rate in this research is quite high (Meiswinkel *et al*., 2008). This may indicate that midges have been feeding on livestock at the collection sites within Mnisi. In case BTV is present within livestock quite a large quantity of *Culicoides* species might possibly be exposed to BTV. However, this does not mean that all species are able to transmit BTV to other livestock. This issue will be explained in more detail in the next section.

Realtime qRT-PCR results

The qRT-PCR tested positive for BTV segment 10 in 63 out of 82 midge pools. A total of 19 samples did not show amplification. In case a midge pool tested positive for BTV, it is unknown how many midges within the pool were positive. Theoretically, just one midge or all midges could have been positive. Provided that the pool size is such that both positive and negative pools are present amongst the total number of pools tested, the field infection prevalence can be calculated using the following formula (Chiang and Reeves, 1962):

$P = 1 - [(n-x)/n]^{1/m} \times 100$

P = estimate of the infection rate

m = pool size

n = number of pools tested

x = number of positive pools

According to this formula, the infection rate of BTV in *Culicoides* species in this study would be estimated at 0,73 (m=200; n=82; x=63).

The collection of an individual *Culicoides* midge by means of a light trap near animals only indicate that it was in close proximity with the animals and does not indicate that a midge was feeding on those animals. As is indicated by Gerry *et al*. (2009), collection of *Culicoides* species by means of light trap collection does not always reflect biting range on animals. In addition, a midge that tested positive in this study is not necessarily vector competent and able to transmit BTV, although species (such as *C. imicola* and *C. bolitinos*) are identified that have been demonstrated to be vector competent in previous studies (Venter *et al*., 1998). A positive midge may carry the virus mechanically or it might have picked up BTV during a previous blood meal from an infected animal, whilst the virus is not able to replicate inside this specific *Culicoides* species (i.e. it is not a vector competent *Culicoides* species). The role in the epidemiology of BT of qRT-PCR positive midges thus remains questionable, even though it might be tested positive. Positive PCR results show that BTV is present within the research area and is not an indication that vector competent *Culicoides* species are present that are capable of transmitting BT to other livestock. Therefore, further research involving species identification and virus isolation of individual BTV-positive midges as well as oral susceptibility studies is needed to resolve the question whether vector competent *Culicoides* species are present within Mnisi (Venter *et al*., 2005).

## Conclusion

A total of 18 561 midges were captured. After differentiation of 1 614 midges (8.7% of total midges caught) by an entomologist 20 species of *Culicoides* were recognized. *C. imicola,* a vector-competent species, was the dominant midge species found in this study. A total of 48,5% of all midges are either parous (26,6%), bloodfed (0,7%) or gravid (21,2%), with the remaining being nulliparous (35,4%) or male (16%). Parity rates found in this research are quite high, which serves as an indicator of increased survival rates in a population. Therefore, midges might have been feeding frequently on nearby livestock. The qRT-PCR tested positive for BTV segment 10 in 63 out of 82 midge pools. A total of 19 samples did not show amplification. The infection rate of BT in *Culicoides* species in this study is estimated to be 0,73. These results show that BTV is present within Mnisi. However, positive qRT-PCR results do not reflect that these species are vector competent *Culicoides* species and are able to transmit BT to other animals. To address the question whether vector competent *Culicoides* midges are present within Mnisi, virus isolations of individual BTV-positive midges and oral susceptibility studies are indicated.

## Further research

Although extensive research has been performed and much is known about BTV and vectors, there are still questions in the BTV research field that need to be clarified. As stated in the introduction, the exact role of certain transmission routes in the epidemiology of BTV remains uncertain. Because of the lack of sufficient monitoring of BTV in some parts of the world, in Asia for example, it is difficult to interpret the BTV status in those areas. To obtain a worldwide overview of the occurrence of specific BTV serotypes and the presence of vector competent *Culicoides* species, sequential identification of BTV and vector analysis throughout the world is necessary, with the emphasis on those parts of the world where no information is available.

This study attempted to map the presence of specific *Culicoides* species and occurrence of BTV in Mnisi, Mpumalanga Province, South Africa. Positive qRT-PCR results show that BTV is present within the research area. However, these positive results are not an indication that vector competent *Culicoides* species are present to transmit BT to other animals. Therefore, further research involving species identification and virus isolation of individual BTV-positive midges and oral susceptibility studies are needed to resolve the question whether vector competent *Culicoides* species are present within Mnisi.

## Abbreviations

BT: bluetongue; BTV: bluetongue virus; BTV-8: bluetongue virus serotype 8.

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## Appendix A

### A.1 Description of trap sites in Mnisi, Mpumalanga Province, South Africa

| Trap Site | Village | Description site | Animals | Distance to fence of kraal | Trap height | Important comments |
|---|---|---|---|---|---|---|
| 1 | Anthol | Trap in tree | Cattle | 2m | 1,5m | |
| 2 | Anthol | Trap on wall of shop | Cattle | 2m | 2m | |
| 3 | Welverdiend | Amanda's home | Cattle and goats | 19 June: unknown because of large kraal<br>26+27 June: 0m<br>03+04 June: 0m | 2,5m | |
| 4 | Welverdiend | Amanda's neighbour | Cattle | 19 June: 7m<br>26+27 June: 4m | 1,5m | |
| 5 | Ludlow | Sister Stanley | Cattle and goats | 0m to cattle<br>1m to goats | 2m | Water nearby |
| 6 | Ludlow | Nice farmer | Cattle | 0m | 1,5m | |
| 7 | Hlalakahle | Big house | Cattle | 0m | 1,5m | |
| 8 | Hlalakahle | | Cattle and donkeys | 0m to cattle<br>10m to donkeys | 1,5m | |
| 9 | Hluvukani | Left house | Cattle | 0m | 1,5m | |
| 10 | Hluvukani | Right house | Cattle | 0m | 1,5m | Big kraal, distance to animals unknown |

## A.2 Logbook of midge collection between 18.06.2013 and 09.07.2013

| Date | Site | Village | Climate<br>Day/night, moon phase | Comments |
|---|---|---|---|---|
| 18.06.2013 | 1 +2 | Anthol | 17/01 °C, half moon | |
| | 5 + 6 | Ludlow | 17/01 °C | Site 5: no cattle inside |
| 19.06.2013 | 3 + 4 | Welverdiend | 22/01 °C | Site 4: trap 7m from cattle |
| | 7 + 8 | Hlalakahle | 23/01 °C | |
| 20.06.2013 | 1 + 2 | Anthol | 20/00 °C, ¾ moon | |
| | 5 + 6 | Ludlow | 20/00 °C | Site 5: big catch |
| 24.06.2013 | 1 + 2 | Anthol | 21/02 °C, full moon | |
| | 5 + 6 | Ludlow | 21/02 °C | Site 5: no cattle inside |
| 25.06.2013 | 1 + 2 | Anthol | 21/03 °C | |
| | 5 + 6 | Ludlow | 21/03 °C | Site 5 + 6: for 4 hours no electricity at night |
| 26.06.2013 | 3 + 4 | Welverdiend | 25/04 °C | Site 3: change of trapsite towards fence of kraal<br>Site 4: trap 4m from cattle |
| | 7 + 8 | Hlalakahle | 24/03 °C | |
| 27. 06.2013 | 3 + 4 | Welverdiend | 23/01 °C, ¾ moon | |
| | 7 + 8 | Hlalakahle | 23/02 °C | |
| 01.07.2013 | 1 + 2 | Anthol | 21/04 °C, half moon<br>Very cloudy on Tuesday morning | Small catch |
| | 5 + 6 | Ludlow | 21/04 °C<br>Very cloudy on Tuesday morning | Small catch |
| 02.07.2013 | 1 + 2 | Anthol | 21/04 °C | |
| | 5 + 6 | Ludlow | 21/04 °C | Site 5: big catch |
| 03.07.2013 | 3 + 4 | Welverdiend | 27/09 °C, ¼ moon | Site 4: trap besides fence from kraal |
| | 7 + 8 | Hlalakahle | 27/09 °C | |
| 04.07.2013 | 3 + 4 | Welverdiend | 25/03 °C | |
| | 7 + 8 | Hlalakahle | 24/02 °C | |
| 08.07.2013 | 9 + 10 | Hluvukani | 19/04 °C, no moon | Site 10: big kraal, cattle could be far away |
| 09.07.2013 | 9 + 10 | Hluvukani | 18/03 °C | Site 10: big kraal, cattle could be far away |

## Appendix B

### B.1 Total RNA extraction from serum or organs using TRI-reagent LS

1. Homogenise sample in 900 µl Trizol in a 2ml tube, mix well and let stand for 5 minutes
2. Add 200 µl Chloroform to each sample, mix well and let stand for 5 minutes
3. Separate phases in a Eppendorf centrifuge pre-cooled to 4 °C by centrifugation at 14.000 rpm for 15 minutes

### B.2 Purification of RNA from TRI-reagent extracts using Qiagen RNeasy Minelute columns (procedure per sample)

1. Prepare Master Mix (volumes for 14 samples) and mix well:
   3,5 ml of buffer RLT with 2,5 ml Ethanol
2. Aliquot 400 µl of Mastermix in 2.0ml Eppendorf tube
3. Add 200 µl of the supernatans prepared according to Appendix A.1 and mix well by pipetting up and down for several times (do not centrifuge)
4. Add the mixture of supernatant and Mastermix to a RNeasy Minelute spin column and centrifuge at maximum speed for 30 seconds
5. Add each spin column to a new 2 ml collection tube (without lid)
6. Add 500 µl buffer RPE
7. Centrifuge for 30 seconds
8. Add each spin column to a new 2 ml collection tube (without lid)
9. Add 500 µl 80% Ethanol
10. Centrifuge for 2 minutes
11. Add each spin column to a new 2 ml collection tube, open the lid of each tube
12. Centrifuge for 5 minutes
13. Add each spin column to a 2 ml collection tube
14. Elute RNA in 15 µl Elution buffer
15. Centrifuge for 1 minute
16. Aliquot RNA into 3 tubes containing 5 µl of RNA
17. Use one for PCR, one for Equine (African Horse Sickness) and one as back up
18. Use immediately or store at -70 °C

## Appendix C

### C.1 Protocol for preparing conventional PCR: gel electrophoresis (1% agarose gel)

1. Dilute 100 ml TBE buffer in 900 ml of water in glass cup
2. Mix 1 g agarose with diluted TBE buffer
3. Melt in microwave until no crystals are seen anymore
4. Cool down the glass with running water until the mixture is lukewarm
5. Add 5 µl effidiumbromide using gloves (this will stick to DNA and makes it orange)
6. Pour gel into essembly using the desired number of wells, make sure there are no bubbles
7. Wait 30 minutes to set the gel
8. Spin gel 90 degrees so that the wells are at the negative site (in black) of the machine
9. Pour buffer over gel
10. Put multiple drops of 1 µl of loading dye on paraffine film corresponding to the number of wells used
11. Add 5 µl of 100 bp marker, negative control, positive control or sample to a drop of loading dye
12. Put each mixture in the correct well
13. Be sure careful documentation of which well contains which control/sample is performed
14. Connect electrodes and switch on machine at 120V

## Appendix D

D.1 Total number of midges caught per trap site, number of midges per pool and differentiation results (species, gender and parity) per trap site

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Trapsite:<br>Other insects:<br>Samples: | 1.1w<br>± 800<br>1.1w: 200 midges<br>1.2w: 188 midges<br><br>Total: 388 midges | | | | | | |
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 86 | 44 | | 6 | 8 | 144 | 72,0 |
| *C. bolitinos* | 6 | 5 | | 3 | | 14 | 7,0 |
| *C. leucostictus* | 1 | 2 | | 1 | 7 | 11 | 5,5 |
| *C. pycnostictus* | 1 | 1 | | | 7 | 9 | 4,5 |
| *C. tropicalis* | 1 | | | 2 | 1 | 4 | 2,0 |
| *C. similis* | 2 | 1 | | | 1 | 4 | 2,0 |
| *C. subschultzei* | | 1 | | | 3 | 4 | 2,0 |
| *C. expectator* | 1 | | | 1 | | 2 | 1,0 |
| *C. nivosus* | | | | 1 | 1 | 2 | 1,0 |
| *C. enderleini* | 1 | 1 | | | | 2 | 1,0 |
| *C. neville* | 1 | | | | | 1 | 0,5 |
| *C. bedfordi* | | 1 | | | | 1 | 0,5 |
| *Species 54 DF* | | 1 | | | | 1 | 0,5 |
| *C. tuttifrutti* | | 1 | | | | 1 | 0,5 |

| | |
|---|---|
| Total | 200 |

| Trapsite:<br>Other insects:<br>Samples | 2.1w<br>± 1000<br>2.1w: 198 midges<br>2.2w: 138 midges<br><br>Total: 336 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 26 | 13 | | 19 | 2 | 60 | 30,3 |
| *C. leucostictus* | 1 | 11 | | 9 | 15 | 36 | 18,2 |
| *C. expectator* | 6 | | | 18 | 3 | 27 | 13,6 |
| *C. tropicalis* | 3 | 1 | | 15 | 6 | 25 | 12,6 |
| *C. similis* | 5 | 1 | | 2 | 5 | 13 | 6,6 |
| *C. bolitinos* | 2 | 4 | | 3 | 1 | 10 | 5,0 |
| *C. tutttifruitti* | | 1 | | 5 | 1 | 7 | 3,5 |
| *C. subschultzei* | | | | 3 | 4 | 7 | 3,5 |
| *C. nivosus* | | | | 1 | 4 | 5 | 2,5 |
| *C. pycnostictus* | 1 | | | | 2 | 3 | 1,5 |
| *C. enderleini* | 1 | | | | 1 | 2 | 1,0 |
| *Species 54 DF* | | 1 | | | | 1 | 0,5 |
| *C. zulu* | | | | | 1 | 1 | 0,5 |
| *C. neavei* | | | | | 1 | 1 | 0,5 |

| Total | 198 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 3.1w<br>± 500<br>3.1w: 193 midges<br>3.2w: 200 midges<br>3.3w: 217 midges<br><br>Total: 610 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 50 | 15 | | 9 | 3 | 77 | 39,9 |
| *C. neville* | 1 | 2 | | | | 3 | 16,0 |
| *C. leucostictus* | 1 | 1 | | 18 | 8 | 28 | 14,5 |
| *C. tutttifruitti* | 13 | 6 | | 7 | 1 | 27 | 14,0 |
| *C. tropicalis* | | | | 15 | 1 | 16 | 8,3 |
| *C. expectator* | 1 | | | 8 | 1 | 10 | 5,2 |
| *C. bolitinos* | 1 | 3 | | 4 | | 8 | 4,1 |
| *C. subschultzei* | 3 | 1 | | 2 | 2 | 8 | 4,1 |
| *C. nivosus* | | 1 | | 5 | 1 | 7 | 3,6 |
| *C. similis* | 1 | 1 | | 1 | 1 | 4 | 2,1 |
| *C. pycnostictus* | | | | | 3 | 3 | 1,6 |
| *C. enderleini* | | 1 | | 1 | | 2 | 1,0 |

| Total: | 193 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 4.1w<br>± 1000<br>4.1w: 176 midges<br><br>Total: 176 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 15 | 15 | | 16 | 9 | 55 | 31,3 |
| *C. leucostictus* | 1 | 4 | | 16 | 22 | 43 | 24,4 |
| *C. tutttifruitti* | 4 | 1 | | 11 | 17 | 33 | 18,8 |
| *C. nivosus* | | | | 5 | 8 | 13 | 7,4 |
| *C. subschultzei* | | | | 1 | 5 | 6 | 3,4 |
| *C. bolitinos* | 2 | 1 | | | 2 | 5 | 2,8 |
| *C. pycnostictus* | 1 | 1 | | 1 | 2 | 5 | 2,8 |
| *C. tropicalis* | | | | 2 | 3 | 5 | 2,8 |
| *C. similis* | | | | 1 | 2 | 3 | 1,7 |
| *Species 54 DF* | | 1 | | 2 | | 3 | 1,7 |
| *C. expectator* | | | | | 2 | 2 | 1,1 |
| *C. bedfordi* | | 1 | | | | 1 | 0,6 |
| *C. neavei* | 1 | | | | | 1 | 0,6 |
| *C. enderleini* | | | | 1 | | 1 | 0,6 |

| Total: | 176 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 5.1w<br>± 2000<br>Test samples 1 – 5w = 200 midges<br>5.1w = 195 midges<br>5.2w – 5.69w = 200 midges<br>5.70w = 116 midges<br><br>Total: 14 916 midges |
|---|---|

| | Females | | | | | | |
|---|---|---|---|---|---|---|---|
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 100 | 48 | | 4 | 6 | 158 | 80,6 |
| *C. nevilli* | 2 | 4 | | 1 | | 7 | 3,6 |
| *C. bedfordi* | 2 | 1 | | 1 | 2 | 6 | 3,1 |
| *C. bolitinos* | 3 | 2 | | 1 | | 6 | 1,5 |
| *C. tropicalis* | | 2 | | 1 | | 3 | 1,0 |
| *C. similis* | 1 | | | 1 | | 2 | 1,0 |
| *C. enderleini* | | 2 | | | | 2 | 1,0 |
| *C. subschultzei* | | 1 | | | 1 | 2 | 1,0 |
| *C. neavei* | | 2 | | | | 2 | 1,0 |
| *C. leucostictus* | 1 | | | | 1 | 2 | 1,0 |
| *C. tutttifruitti* | | | | 2 | | 2 | 1,0 |
| *C. zuluensis* | 1 | | | | | 1 | 0,5 |
| *C. expectator* | 1 | | | | | 1 | 0,5 |
| *C. coavtatus* | 1 | | | | | 1 | 0,5 |
| *C. brucei* | | | | 1 | | 1 | 0,5 |

| Total: | 196 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 6.1w<br>± 800<br>6.1w: 199 midges<br>6.2w-6.8w: 200 midges<br>6.9w: 83 midges<br><br>Total: 1 683 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 78 | 45 | 1 | 2 | 6 | 132 | 66,3 |
| *C. bedfordi* | 3 | | 1 | 1 | 5 | 10 | 5,0 |
| *C. neavei* | 4 | 1 | | | 5 | 10 | 5,0 |
| *C. nevilli* | 3 | 1 | | | 2 | 6 | 3,0 |
| *C. brucei* | 3 | 1 | | | 1 | 5 | 2,5 |
| *C. carctatus* | 3 | 1 | | | 1 | 5 | 2,5 |
| *C. leucostictus* | 1 | 1 | | | 2 | 4 | 2,0 |
| *C. similis* | | 2 | | 1 | 1 | 4 | 2,0 |
| *C. tutttifruitti* | 2 | 1 | | | 1 | 4 | 2,0 |
| *C. subschultzei* | 2 | 1 | | | 1 | 4 | 2,0 |
| *C. tropicalis* | 1 | | | 1 | 1 | 3 | 1,5 |
| *C. expectator* | | | | 3 | | 3 | 1,5 |
| *C. bolitinos* | 1 | 1 | | 1 | | 3 | 1,5 |
| *C. pycnostictis* | 1 | | | | 1 | 2 | 1,0 |
| *Species 54 DF* | 1 | | | | | 1 | 0,5 |
| *C. zuluensis* | 1 | | | | | 1 | 0,5 |
| *C. nivosus* | 1 | | | | | 1 | 0,5 |
| *C. dekeyseri* | 1 | | | | | 1 | 0,5 |

| Total: | 199 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 7.1w<br>± 300<br>7.1w: 109 midges<br><br>Total: 109 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 26 | 6 | | 6 | 1 | 39 | 35,8 |
| *C. tropicalis* | | 1 | | 16 | | 17 | 15,6 |
| *C. bolitinos* | 4 | 5 | | 4 | 1 | 14 | 12,8 |
| *Species 54 DF* | 1 | 3 | | 6 | | 10 | 9,2 |
| *C. leucostictus* | 4 | | | 3 | | 7 | 6,4 |
| *C. pycnostictus* | 2 | | | 1 | 1 | 4 | 3,7 |
| *C. tutttifruitti* | 1 | | | 2 | 1 | 4 | 3,7 |
| *C. expectator* | | | | 3 | | 3 | 2,8 |
| *C. nivosus* | | 1 | | 1 | 1 | 3 | 2,8 |
| *C. enderleini* | 1 | 1 | | | | 2 | 1,8 |
| *C. similis* | | 1 | | 1 | | 2 | 1,8 |
| *C. bedfordi* | 1 | | | 1 | | 2 | 1,8 |
| *C. glabripernis* | | | | 1 | | 1 | 0,9 |
| *C. zuluensis* | | | | 1 | | 1 | 0,9 |

| Total: | 109 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 8.1w<br>± 600<br>8.1w: 120 midges<br><br>Total: 120 midges | | | | | | |
|---|---|---|---|---|---|---|---|
| | Females | | | | | | |
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 18 | 25 | | 5 | 1 | 49 | 40.8 |
| *C. tropicanis* | 1 | 2 | | 13 | 1 | 17 | 14,2 |
| *C. bolitinos* | 1 | 6 | | 1 | 3 | 11 | 9,2 |
| *C. leucostictus* | 3 | 3 | | 2 | 2 | 10 | 8,3 |
| *Species 54 DF* | | 4 | | 5 | | 9 | 7,5 |
| *C. similis* | 3 | | | 1 | 2 | 6 | 5,0 |
| *C. expectator* | | | | 6 | | 6 | 5,0 |
| *C. tutttifruitti* | 1 | 1 | | 2 | | 4 | 3,3 |
| *C. enderleini* | 1 | 1 | | | 1 | 3 | 2,5 |
| *C. pycnostictus* | | | | 1 | 2 | 3 | 2,5 |
| *C. nivosus* | | | | 1 | | 1 | 0,8 |
| *C. bedfordi* | | 1 | | | | 1 | 0,8 |

| Total: | 120 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 9.1w<br>± 600<br>9.1w: 152 midges<br><br>Total: 152 midges |
|---|---|

| | Females | | | | | | |
|---|---|---|---|---|---|---|---|
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 20 | 42 | 3 | 10 | 6 | 81 | 53,3 |
| *C. leucostictus* | 1 | 6 | 4 | 5 | 12 | 28 | 18,4 |
| *C. tutttifruitti* | | 7 | | 1 | 1 | 9 | 5,9 |
| *C. nivosus* | | | | | 5 | 5 | 3,3 |
| *C. bolitinos* | 2 | 2 | | | 1 | 5 | 3,3 |
| *C. subschultzei* | 1 | 3 | | | 1 | 5 | 3,3 |
| *C. similis* | | 2 | | 1 | 2 | 5 | 3,3 |
| *C. pycnostictus* | | 1 | | 1 | 2 | 4 | 2,6 |
| *C. coarctatus* | 2 | | | | 1 | 3 | 2,0 |
| *C. bedfordi* | | 1 | | 1 | | 2 | 1,3 |
| *C. tropicanis* | | 1 | | 1 | | 2 | 1,3 |
| *C. enderleini* | | 1 | | | | 1 | 0,7 |
| *C. neavei* | | 1 | | | | 1 | 0,7 |
| *C. expectator* | 1 | | | | | 1 | 0,7 |

| Total: | 152 |
|---|---|

| Trapsite:<br>Other insects:<br>Samples | 10.1w<br>± 400<br>101w: 71 midges<br><br>Total: 71 midges |
|---|---|

| | Females | | | | | | |
|---|---|---|---|---|---|---|---|
| *Culicoides* species | Nulliparous | Parous | Bloodfed | Gravid | Males | Total | % |
| *C. imicola* | 12 | 26 | 2 | 1 | 1 | 42 | 59,2 |
| *C. leucostictus* | 1 | 2 | 1 | 1 | 3 | 8 | 11,3 |
| *C. tutttifruitti* | 3 | 4 | | 1 | | 8 | 11,3 |
| *C. expectator* | | 4 | | | | 4 | 5,6 |
| *C. bolitinos* | 1 | | | | 2 | 3 | 4,2 |
| *Species 54 DF* | | 2 | | | | 2 | 2,8 |
| *C. nivosus* | | 1 | | | 1 | 2 | 2,8 |
| *C. subschultzei* | | 1 | | | | 1 | 1,4 |
| *C. tropicalis* | 1 | | | | | 1 | 1,4 |

| Total: | 71 |
|---|---|