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Investigation of selected determinants of *Culicoides* vectorial capacity of African horse sickness at Onderstepoort, South Africa

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Investigation of selected determinants of *Culicoides* vectorial capacity for African horse sickness at Onderstepoort, South Africa

Für meine Eltern

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Abbreviations

AHS	African horse sickness
AHSV	African horse sickness virus
BT	Bluetongue
BTV	Bluetongue virus
CDC	Centre for Disease Control
CO_2	carbon-dioxide
C_T	cycle threshold
D_0	day of feeding
D_{10}	10 days after feeding
DIVA	differentiation of infected and vaccinated animals
EE	Equine encephalosis
EEV	Equine encephalosis virus
EIBH	equine insect bite hypersensitivity
EIP	extrinsic incubation period
ELISA	enzyme-linked immunosorbent assay
ENSO	El Niño/Southern Oscillation
MEB	mesenteron escape barrier
MIB	mesenteron infection barrier
OIE	Office International des Epizooties (World Organisation for Animal Health)
OVAH	Onderstepoort Veterinary Academic Hospital
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT-qPCR	reverse transcription quantitative polymerase chain reaction
rRT-PCR	real-time reverse transcription polymerase chain reaction
UV	ultraviolet
WNV	West Nile virus

Untersuchung ausgewählter Determinanten der vektoriellen Kapazität von *Culicoides* für Afrikanische Pferdepest in Onderstepoort, Südafrika

Zusammenfassung

Culicoides Stechmücken (Diptera: Ceratopogonidae) sind Vektoren für eine Vielzahl von Pathogenen, unter anderem für das Virus der Afrikanischen Pferdepest (AHSV), das zum *Orbivirus* Genus aus der Familie der *Reoviridae* zählt. Dieses Virus verursacht Afrikanische Pferdepest, eine Erkrankung von Equiden, die in der Subsahara endemisch vorkommt und eine ausgesprochen hohe Mortalität aufweist. *Culicoides* (*Avaritia*) *imicola* Kieffer wird als Hauptvektor für AHSV betrachtet und ist die dominierende *Culicoides*-Spezies in Südafrika. Aufgrund der globalen Verteilung von Stechmücken besteht die Gefahr, dass sich die Krankheit auch ausserhalb ihrer bekannten Grenzen ausbreitet, was enorme ökonomische Auswirkungen auf die Pferdeindustrie hätte. Als Teil der Gefahrenanalyse ist es essentiell, dokumentierte und bislang undokumentierte, potentiell neue Vektoren zu erforschen. Die vorliegende Studie hat zwei Fangmethoden für *Culicoides* Stechmücken verglichen: die konventionelle sogenannte "Onderstepoort-Lichtfalle", die über Nacht betrieben wird, und die mechanische Aspiration von *Culicoides* direkt von Pferden bei Sonnenuntergang. *Culicoides imicola* stellte die überwiegende Spezies in beiden Fangmethoden dar. Andere Spezies, hauptsächlich *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel und *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro, waren in der Lichtfalle deutlich unterrepräsentiert; stellten aber einen wesentlichen Anteil in der mechanischen Aspiration dar. Optimale Ergebnisse wurden je nach Fangmethode zu unterschiedlichen Zeiten erreicht. Die mechanische Aspiration stellt eine nützliche Erweiterung zu der Lichtfalle dar und ist womöglich sogar die bessere Wahl für die Erforschung von Vektoren. Quantitative Real-time Reverse Transkriptase-Polymerase-Kettenreaktion (RT-qPCR) der gesammelten *Culicoides* Stechmücken ergab eine Infektions-rate von 1.14%. Virus war nicht nur in Pools von Stechmücken aus der Lichtfalle, sondern auch aus den Pools nach mechanischer Aspiration präsent. Sieben der AHSV-positiven Pools beinhalteten nur *C. imicola*, vier Pools bestanden aus verschiedenen Spezies und ein Pool schloss *C. imicola* aus, was das Vorkommen einer weiteren Vektorspezies vermuten lässt.

In einem separaten Teil der Studie wurden *C. imicola* Stechmücken, die im Freien gefangen wurden, mit AHSV-positivem Blut gefüttert. Einzelne Individuen wurden in Kopf und

Abdomen seziert und Real-time RT-qPCR dieser Teile direkt nach der Blutmahlzeit und nach 10-tägiger Inkubation durchgeführt. Während der weit überwiegende Anteil der *Culicoides* direkt nach dem Blutmahl AHSV-positiv waren (95.7%), konnte das Virus nach 10-tägiger Inkubation nur noch in 51% der Mücken dargestellt werden. Interessanterweise war eine signifikant höhere Anzahl von Mücken im Abdomen aber nicht im Kopfteil positiv für AHSV. Daraus lässt sich schlussfolgern, dass *C. imicola* – genau wie andere *Culicoides* Arten – über ein sogenanntes "Mesenteronales Escape Barrier" verfügt, d.h. Virusreplikation findet statt, aber die Viruspartikel können den Mitteldarm nicht verlassen. Die durchschnittliche Virusmenge pro Stechmücke erhöhte sich während der 10-tägigen Inkubation, vermutlich aufgrund von Virusreplikation im Abdomen. Replikation in den Speicheldrüsen konnte nicht dargestellt werden. Der entwickelte RT-qPCR-Assay hat sich als sehr praktische Methode zur Untersuchung von sowohl von *Culicoides*-Pools als auch von einzelnen Mücken erwiesen.

Summary

Culicoides biting midges (Diptera: Ceratopogonidae) are vectors of a variety of pathogens including African horse sickness virus (AHSV), a member of the *Orbivirus* genus in the *Reoviridae* family. AHSV causes African horse sickness (AHS), a disease of equids endemic in sub-Saharan Africa with an extremely high mortality rate. *Culicoides* (*Avaritia*) *imicola* Kieffer is considered to be the principal vector for AHSV and is the dominant *Culicoides* species in South Africa. Due to the global distribution of the vector species, the disease is at risk of spreading outside its traditional boundaries, which could have a severe economical impact on the equine industry. As part of the risk assessment it is essential to monitor known vectors as well as potential vector species. The present study compared two trapping methods for *Culicoides* midges. The conventional Onderstepoort light trap that was operated overnight was compared to mechanical aspiration from bait horses at sunset. *Culicoides imicola* was confirmed as the predominant species by both trapping methods. Other species, mainly *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel and *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro, were highly underrepresented in the light trap collections, but made a significant contribution to the mechanical aspiration catches. The time for optimal collection also differed between both trapping devices, leading to the conclusion that mechanical aspiration is a useful addition to conventional light trap collection and possibly the better choice when investigating insect vectors. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) of collected *Culicoides* midges showed an infection rate of 1.14%, exceeding previous estimates. Virus was present in midge pools collected from the light trap as well as the mechanical aspiration. Seven of the positive pools consisted only of *C. imicola*, four contained mixed species and one pool contained no *C. imicola*, suggesting the presence of another vector species.

In a separate part of the study, field-collected *C. imicola* were fed with AHSV-positive blood. Individual midges were dissected and real-time RT-qPCR conducted on heads/thoraxes and abdomens immediately after feeding and after 10 days of incubation. While the majority of *Culicoides* were AHSV-positive directly after feeding (95.7%), virus was still present in 51% of the midges after 10 days of incubation.Significantly more midges were AHSV-positive in the abdomen compared to heads/thoraxes, indicating that *C. imicola* – like other *Culicoides* species – express a so-called mesenteronal escape barrier (MEB), i.e. virus replicates, but is unable to disseminate from the midgut of the midge. The mean amount of virus in the midges increased after 10 days of incubation, most likely due to viral replication in the abdomen.

Replication in the salivary glands could not be shown. The RT-qPCR assay proved useful for investigation of midge pools as well as individual *Culicoides* midges.

Chapter One: Literature Review

African horse sickness

African horse sickness (AHS) is an infectious, non-contagious disease of equids transmitted by *Culicoides* biting midges. The disease is endemic in sub-Saharan Africa where it presents itself with a seasonal occurrence. In South Africa it is known to spread from the north-eastern parts of the country, the severity of outbreaks depending on the time of the year and the climatic conditions. Although the Sahara Desert and the Mediterranean Sea form natural borders limiting the spread of AHS, the disease has occasionally extended from northern Africa into the Middle East and Spain. After the first European outbreak in 1966, AHS was reintroduced into Spain in 1987, when it was associated with subclinically infected zebras that had been imported from Namibia (Lubroth, 1988). In contrast to expectations that the virus would be unable to overwinter, the outbeak lasted until 1991. Due to its severity, its ability to spread rapidly and its importance for the international trade of animals, the disease has been allocated a listed status by the World Organisation for Animal Health (OIE) (2010).

All equids are susceptible to AHS, but with 70 – 95% horses show the highest mortality rate (Coetzer and Guthrie, 2004). The early death of the host limits the extent of possible viral transmission, which is why horses are regarded as incidental hosts (Guthrie and Quan, 2009; Wilson et al., 2009). Zebras *(Equus burchelli)* only show subclinical infection, but may play a vital role in the persistence of the virus in an area. They are considered reservoir hosts and are essential for the endemic circulation of African horse sickness virus (AHSV) (Wilson et al., 2009). A continuous transmission cycle of virus has been shown between *Culicoides* midges and zebras in the Kruger National Park in South Africa (Barnard, 1993). The consistent reduction of zebra population numbers in South Africa over the past decades is hypothesized to lower the incidence of AHS cases during that time (Barnard, 1998). Similarly, areas of northern Africa without residing zebra populations do not show persistent AHS occurrence (Mellor and Hamblin, 2004). However, zebras are not essential for virus replication and the transmission cycle (Wilson et al., 2009). African donkeys also only become subclinically infected and may play a similar role to zebras in areas with large donkey populations (Hamblin et al., 1998). Dogs and certain other carnivores may be infected by ingestion of infected meat, resulting in severe clinical disease and frequently even death of affected animals (Wilson et al., 2009). They are also susceptible to infection by inoculation of virulent horse blood and the disease has been experimentally shown to be transmitted between dogs and from a dog to a

horse (Theiler, 1906). However, transmission of AHS from a carnivore to an equid has yet to be shown in the field. Additionally, it is unknown whether *Culicoides* midges even feed on dogs. Using a mechanical aspirator on a dog in Israel, Braverman and Chizov-Ginzburg (1996) could not locate any *Culicoides* midges. Light trap collections near a dog kennel also showed a significantly smaller number of *Culicoides* than other light trap collections and all investigated blood meals were negative for canine blood (Braverman and Chizov-Ginzburg, 1996). Until proven otherwise, carnivores are therefore not considered relevant for the epidemiology of AHS (Alexander et al., 1995). AHSV infection in camel (*Hyalomma dromedarii*) (Awad et al., 1981) as well as neutralising antibodies in elephant (*Loxodonta africana*) have been described (Binepal et al., 1992). However, these findings seem to be without epidemiological significance.

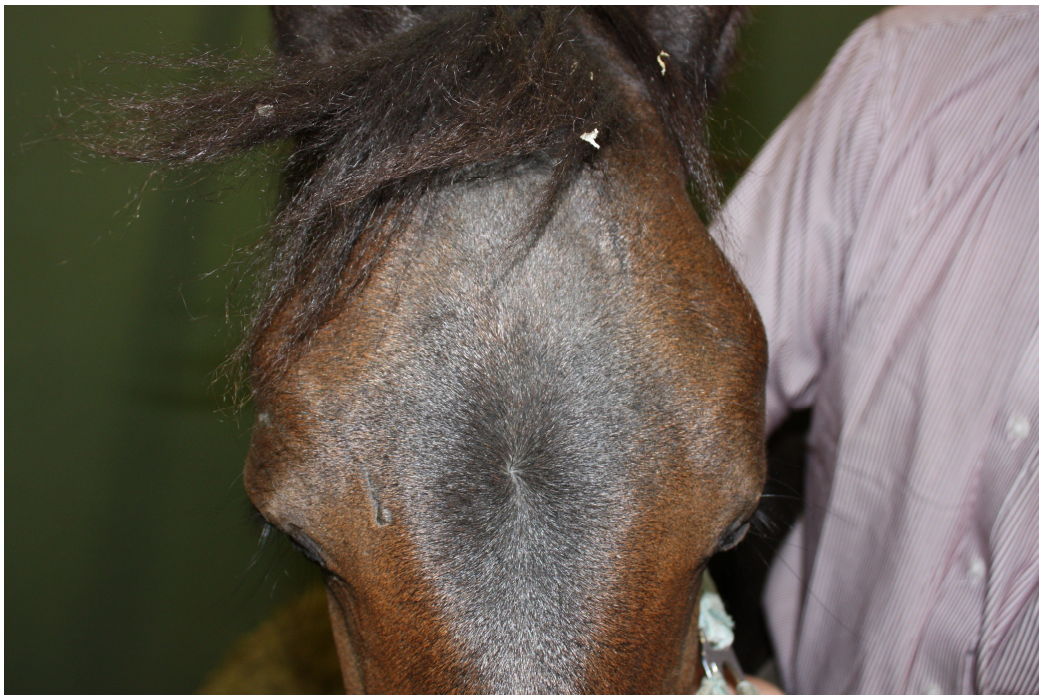

Figure 1: African horse sickness – cardiac form (dikkop): swelling and oedema of the supraorbital fossa. © Alan J. Guthrie

Based on clinical signs, four forms of AHS have been described (Theiler, 1921). Horse owners perceive oedema and fever in their animals. The peracute pulmonary or 'dunkop' form manifests as a high fever along with respiratory signs due to pulmonary oedema and hydrothorax. This form usually has a lethal outcome (mortality rate over 95%) and commonly occurs in fully susceptible, i.e. non-vaccinated, horses as well as in dogs (Coetzer and Guthrie, 2004). The course of disease of the subacute cardiac 'dikkop' form is milder, but still has a mortality rate of 50%. Subcutaneous oedema of the head and neck and particularly of

the supraorbital fossa are characteristic and necropsy usually reveals a hydropericardium. An acute 'mixed' form combining findings of the pulmonary and the cardiac form is possible and shows a mortality rate of 70% (Coetzer and Guthrie, 2004).

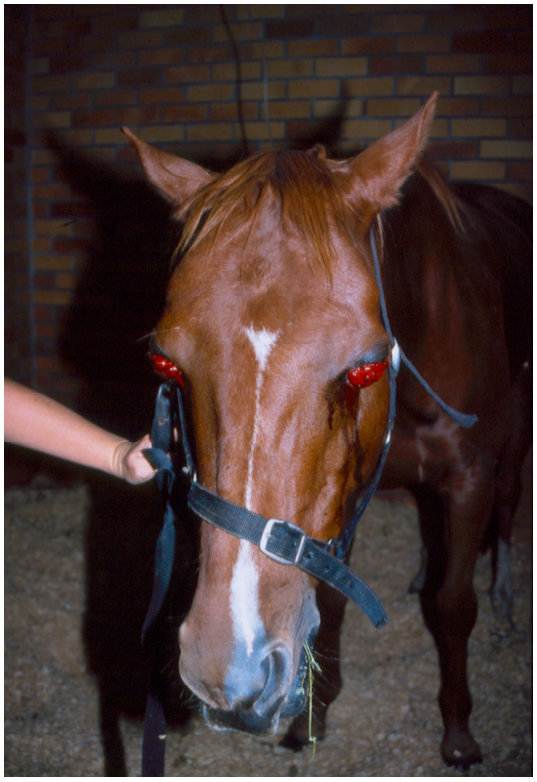

Figure 2: African horse sickness - scleral oedem and haemorrhage. © Alan J. Guthrie

Figure 3: African horse sickness - pulmonary form (dunkop): severe lung oedema. © Alan J. Guthrie

The subclinical 'horse sickness fever' form only leads to mild fever and oedema and horses convalesce quickly. This is the only form seen in wild equids, but it can also occur in vaccinated animals as well as animals infected with a low-virulence strain of the virus. In contrast to Bluetongue virus (BTV) infection, AHS does not cause abortion amongst pregnant animals and there is no indication of vertical transmission (MacLachlan and Guthrie, 2010). Animals that recover from the disease develop a good immunity to the infecting serotype and partially to other serotypes and do not remain carriers of the virus (OIE, 2010).

Since no curative treatment is available for animals suffering from AHS, prevention and control are the key points for managing this disease. Restriction of animal movement can prevent new outbreaks. Animals can still be moved from infected countries into AHS-free countries, if testing and quarantine regulations prescribed by the OIE are strictly adhered to (OIE, 2010). Vector control is important and can be achieved by use of insecticides, larvicides and/or repellents and by modification of husbandry in order to destroy vector habitat and to protect animals from vectors. Regular vaccination of susceptible animals in endemic areas is elementary in controlling a disease and has probably played a crucial role in decreasing clinical cases of AHS in South Africa over the past years (Mellor and Hamblin, 2004). Slaughter of viraemic animals is usually prescribed as part of governmental emergency control in order to stop an epidemic at an early stage. However, because of the emotional and monetary value of horses, this policy is more likely to result in dissemination of the disease by illegal movement of infected animals rather than restriction and/or elimination of the disease. Checking for fever twice daily is recommended to identify infected horses at an early stage.

African horse sickness virus

The causative agent of AHS is AHSV, a member of the genus *Orbivirus* in the *Reoviridae* family (Verwoerd et al., 1979). It is a non-enveloped double-stranded RNA virus with morphological similarities to other orbiviruses such as BTV and Equine encephalosis virus (EEV) (Verwoerd et al., 1979; Calisher and Mertens, 1998). The genome of AHSV is composed of 10 segments of double stranded RNA, encoding 7 structural proteins called VP1 – VP7 and four non-structural proteins called NS1, NS2, NS3 and NS3a (Roy et al., 1994), which together construct the virus and ensure viral replication. Surrounding the genome is a virus core: the inner layer mainly consists of VP3, its outer surface of VP7 and the outer capsid is composed of the two major proteins VP2 and VP5 (Roy et al., 1994). It is the two latter proteins that lead to cell attachment and penetration in mammalian cells (Wilson et al., 2009). They

also induce neutralising antibodies and define the serotype (Mertens et al., 1989; Singh et al., 2004; Maan et al., 2007). The segment encoding VP2 is the most variable of the genome and VP2 is considered the major factor for serotype determination (Maan et al., 2007). VP3 and VP7 on the other hand are highly conserved among the different serotypes (Bremer et al., 1990; Mellor and Hamblin, 2004), one of the reasons reverse transcription polymerase chain reaction (RT-PCR) for diagnostic purposes targets VP7 sequences (Zientara et al., 1993). NS1 is another protein which is highly conserved among serotypes (Rodríguez-Sanchez et al., 2008). The region of the genome of segment 10 encoding for NS3 varies among the AHSV serotypes as NS3 is the second most variable protein after VP2. It is membrane-associated and suggested to play a role in viral morphogenesis and release of virions (Quan et al., 2008).

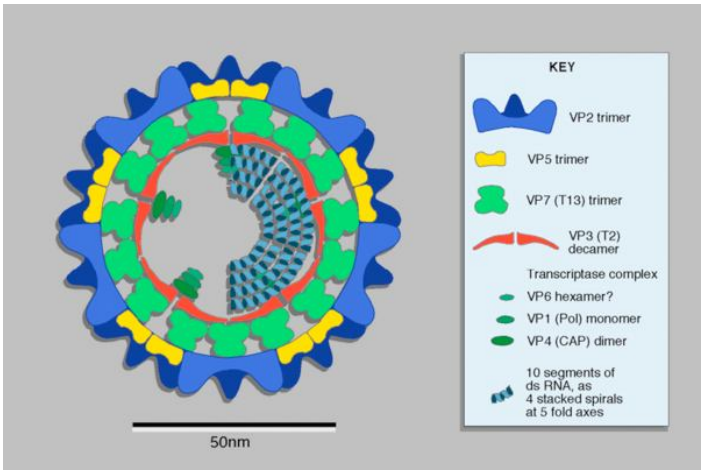

Figure 4: Structure of the African horse sickness virus. © Elsevier

AHSV survives a pH of 6.0 – 12.0, but is inactivated below pH 6.0. Putrid blood can remain infective for over two years. The virus is stable at 4°C, at −70°C and at up to 75°C if stored in the right medium (OIE, 2010), but can become unstable at −25°C (Coetzer and Guthrie, 2004).

Nine serotypes of AHSV have been described (McIntosh, 1958; Howell, 1962), all of which occur in eastern and southern Africa. Serotype 9 is the most widespread and responsible for most of the AHS outbreaks outside of Africa. Exceptions are the epidemic in Spain in 1987 – 1990 which was caused by AHSV-4 (Mellor et al., 1990; Mellor and Hamblin, 2004) and more recent outbreaks which were caused by AHSV-2 and other serotypes (MacLachlan and Guthrie, 2010).

The first effective vaccine against AHS was developed in the 1930s (Alexander and Du Toit, 1934; Alexander, 1936). Currently, a polyvalent live-attenuated vaccine is available and widely used in South Africa. Horse owners are required to vaccinate their animals annually in late winter to early summer. Vaccination does not guarantee full protection against infection or disease, but with each subsequent booster vaccination protection of individual horses increases. The vaccine is supplied in two vials, which have to be given three weeks apart, and cover serotypes 1, 3 and 4 and 2, 6, 7 and 8, respectively (Mellor and Hamblin, 2004). AHSV-5 is not included, because it has led to severe reactions including death in vaccinated animals (von Teichman et al., 2010). In addition, AHSV-8 and AHSV-6 are considered to be cross-protective with serotype 5 and 9, respectively, which is why the latter is also not included in the vaccine. AHSV-9 is considered to be of low virulence and less common in southern Africa (Mellor and Hamblin, 2004). However, it is prevalent in western Africa and responsible for most epidemics outside Africa. Blackburn and Swanepoel (1988) demonstrated that wild virus induces a more broadly cross-reactive immune response than attenuated virus and that repeated immunisation will lead to broader response to the various serotypes and higher individual titres. Best immunity is therefore induced by annual booster vaccinations. Foals acquire passive immunity via colostrum with the level of antibodies dependent on the antibody level of their dams (Blackburn and Swanepoel, 1988). Foals born to immune mares should not be immunized before six months of age so as not to interfere with natural immunity (Coetzer and Guthrie, 2004). Live-attenuated vaccines are cheap and effective in preventing AHS, but also involve risks such as adverse vaccine reactions (including death), variable immune responses and viraemia in inoculated animals, the risk of reversion to virulence and reassortment with wild-type AHSV strains in the field (Paweska and Venter, 2003). For example, when investigating the virus recovery rate for AHSV-7 in the vector species after an incubation period of 10 days, Venter and Paweska (2007) demonstrated higher recovery rates for AHSV-7 vaccine strain than for field isolates of the same serotype. This finding was valid for various species of the vector. Inactivated vaccines are considered safer than live ones, but are more expensive and do not induce immunity as effective and for as long duration. The disadvantages of both kinds of vaccines have led to the drive to develop recombinant AHSV vaccines (Paweska and Venter, 2003). Guthrie et al. (2009) have recently developed a recombinant vaccine for AHSV-4, which appears to be safe and to induce protective immunity effectively. A great advantage if AHS should spread into regions previously free of AHSV is that it allows the differentiation between infected and vaccinated animals (DIVA). This recombinant canarypox virus vectored vaccine uses genes encoding for the outer capsid proteins

VP2 and VP5 of AHSV-4. PCR assays that do not target the genes encoding for these proteins will therefore be able to differentiate vaccinated from naturally infected animals. The canary pox virus has already been used as vector in several commercial vaccines worldwide and continued investigations towards recombinant polyvalent vaccines are advised (Guthrie et al., 2009).

In the mammalian host, AHSV replicates in the regional lymph nodes leading to primary viraemia. After infection of organs including lungs, spleen and other lymphoid tissues, secondary viraemia is of variable duration (Mellor and Hamblin, 2004). The structural proteins VP2 and VP5 enable the virus to attach to and penetrate endothelial cells and leukocytes. Inside mammalian cells, AHSV replicates with the help of its own VP1 polymerase, allowing independent virus replication. After exiting the cell, virus distributes freely in the blood as an infectious particle. This cell-free viraemia can last for four to eight days in horses (Wilson et al., 2009), and is high compared to the viraemia seen in donkeys and zebras, where it can last up to 28 days (Coetzer and Guthrie, 2004). As soon as antibodies are produced by the host, they bind to the epitopes on VP2 and VP5; thus neutralizing the virus particle and ending the viraemic phase. However, remnants of AHSV infection are evident for a much longer period. With the help of a real-time quantitative RT-PCR (RT-qPCR), AHSV RNA can be measured in horses for 97 days post infection (Quan et al., 2010). It has to be pointed out that this method only discovers RNA fragments of AHSV. A positive real time RT-qPCR result therefore only proves previous exposure to AHSV, but does not imply ongoing infection. The incubation period of AHS is less than nine days (Mellor et al., 2004).

AHSV is an arthropod-borne virus (arbovirus). In the vector, the structural protein VP7 is responsible for cell attachment and penetration. Because AHSV uses a different protein to bind to host and vector cells, respectively, it is still infectious in the insect vector even if it has been neutralised by antibodies of the host. Ingested blood is deposited in the hind part of the midgut of the midge, where the virus particles attach to the luminal surface of the gut cells; entering, infecting and replicating in them. Virions are released through the basolateral membrane into the haemocoel, where they are believed to infect secondary target organs like the fat body and salivary glands (Mellor et al., 2000; Wittmann and Baylis, 2000). Following the ingestion of the blood meal the virus titre decreases in the midge during the so-called eclipse phase or partial eclipse phase. In *Culicoides* (*Monoculicoides*) *variipennis* Coquillett, BTV titres rose 10^3 – 10^4-fold and plateau 7 − 9 days post infection. Virus titres are maintained for

the remainder of the vector's life (Mellor, 1990). The interval between virus ingestion and ability of the midge to transmit virus is known as extrinsic incubation period (EIP), which generally takes about 10 days at 25°C (Wittmann and Baylis, 2000).

The EIP, the infection rate of the vector as well as rate of virogenesis are highly dependent on temperature: with rising temperature the infection rate of *Culicoides* biting midges will increase and the replication of AHSV accelerate (Wittmann and Baylis, 2000; Wilson et al., 2009). Decreasing temperatures increase the duration of EIP and replication of the virus will decrease correspondingly until a lower threshold of approximately 15°C is reached, where replication stops completely (Wittmann et al., 2002). The virus can, however, persist in a latent state and resume virogenesis as soon as the threshold is exceeded, which could be shown for *Culicoides* (*Monoculicoides*) *sonorensis* Wirth & Jones and AHSV-9 (Wellby et al., 1996) as well as AHSV-4 (Wittmann et al., 2002). Longevity of adult *C. sonorensis* was shown to decrease with increasing temperature and a significant interaction was noted between temperature and humidity: at low temperatures survival was greater with high relative humidity and vice versa. Temperature and humidity can greatly affect transmission of orbiviruses by *C. sonorensis*. Midge longevity, virus replication rate and the proportion of females capable of taking at least one virus transmissive blood meal increase with temperature (Wittmann et al., 2002). Wittmann et al. (2002) concluded the optimum temperature for virus transmission to be 27 − 30°C or even higher. However, not all *Culicoides* midges are susceptible to infection with AHSV. Possible hurdles are a mesenteron infection barrier (MIB), where virus is unable to enter the midgut cells, a mesenteron escape barrier (MEB), where virus replicates but is unable to exit midgut cells (Jennings and Mellor, 1987) and a dissemination barrier, where virus replicates and enters the haemocoel, but is unable to infect secondary target organs (Fu et al., 1999; Mellor et al., 2000). Jennings and Mellor (1987) found 43.6% of persistently infected *C. variipennis* to express a MEB, concluding a risk of overestimating the vector potential of such populations. A salivary gland infection barrier and a salivary gland escape barrier also have to be passed by arboviruses in order to fully infect haematophagous arthropods. However, they have not been shown to play a role in *Culicoides* species (Mellor, 1990). All barriers mentioned are controlled genetically, but may also be influenced by temperature (Mellor et al., 1998; Wittmann and Baylis, 2000).

AHSV can be transmitted by various other insects such as mosquitoes (*Culex, Anopheles, Aedes* spp.), ticks (*Hyalomma, Rhipicephalus*) and possibly biting flies (*Stomoxys, Tabanus*)

in the laboratory, but apart from *Culicoides* biting midges, none have been show to play a role under natural conditions (Mellor, 1994; OIE, 2010). Horses can be infected by parenteral inoculation of infective blood or organ suspensions with infection occurring more readily by the intravenous than by the subcutaneous route (Theiler, 1921).

Polymerase chain reaction

Before PCR assays for detection of AHSV were developed, AHS infection was diagnosed by intracerebral inoculation of suckling mice, infection of cell cultures or the complement fixation test. However, these techniques have the disadvantages of prolonged processing in the laboratory, leading to a delay in the implementation of sanitary measures by the responsible authorities. Accordingly, alternative and more rapid ways of diagnosing AHSV were examined and a PCR assay developed which is capable of rapidly identifying the various serotypes of the virus (Zientara et al., 1993). Another limitation was that low viraemia was not always detectable by virus isolation (Hess, 1988). In France, Zientara et al. (1993) used primers based on segment 7 of AHSV-4, which encodes for VP7. As mentioned earlier, this protein is well conserved in all nine serotypes. By applying restriction enzyme digestion of the resulting PCR products, differentiation became possible between the viruses AHSV-2, -4, -5, -7 and -9 and the group of serotypes of AHSV-1, -3, -6, and -8 (Zientara et al., 1993). Simultaneously, Stone-Marschat et al. (1994) also developed a RT-PCR in the United States. They targeted the S8 gene of AHSV-9, which encodes for NS2 and is highly conserved within all serotypes of a particular *Orbivirus* but is divergent among the different *Orbivirus* serogroups. Again, all serotypes could be amplified, but other orbiviruses such as BTV and EEV as well as the epizootic hemorrhagic disease virus could not be amplified, indicating serogroup specificity. AHSV could be detected in cell cultures, in blood from viraemic animals and in tissues of animals that died of AHS. This technique could even pick up infection with low levels of viraemia, proving RT-PCR more sensitive than virus isolation (Stone-Marschat et al., 1994). Zientara et al. (1995) compared results of a RT-PCR which used primers for AHSV-4 segment 7 and AHSV-3 segment 10 to those of virus isolation and enzyme-linked immunosorbent assay (ELISA). The results obtained were consistent, except for three samples, which tested negative with ELISA and virus isolation, but positive with RT-PCR. They concluded an improved sensitivity and specificity of the RT-PCR. Because it is also the most rapid technique available it was suggested to complement ELISA or other assays in outbreak investigations (Zientara et al., 1995).

The first serotype-specific RT-PCR for AHSV using nine pairs of primers specifically designed for each serotype was described in 2000 (Sailleau et al., 2000). Genome segment 2, which encodes the serotype-specific protein VP2, was targeted. It was as sensitive and specific as previously published techniques and showed perfect agreement with virus isolation. Samples as old as 37 years, from different geographical regions and of live and formalin-inactivated viruses were successfully evaluated (Sailleau et al., 2000). The first real-time RT-PCR (rRT-PCR) for detection of AHSV was described in 2008 (Rodríguez-Sanchez et al., 2008). Targeting segment 5 encoding for NS1, another region highly conserved among serotypes, investigators tested AHSV reference strains, field viruses as well as BTV reference strains, West Nile virus (WNV), Equine infectious anaemia virus, Equine arteritis virus and blood from a healthy horse. Three different PCR assays were compared: a conventional gel-based RT-PCR, an rRT-PCR with SYBR-Green and an rRT-PCR with a TaqMan® probe. Every assay detected all nine known AHSV serotypes and was specific enough not to detect related viruses. The conventional RT-PCR only requires basic laboratory tools and is sensitive and specific enough to be a useful assay for simple first-line diagnosis. Because no visualization is necessary, the rRT-PCR assays are quicker, saving time and laboratory reagents. However, a more expensive thermocycler is needed. While the TaqMan® rRT-PCR is the quickest, completing a run within two hours, the rRT-PCR using SYBR-Green is the most sensitive test with a detection limit of 0.1 $TCID_{50}$/ml (Rodríguez-Sanchez et al., 2008), whereas other tests need 1.2 $TCID_{50}$/ml. Fernández-Pinero (2009) also described an rRT-PCR for AHSV detection. This test targets segment 7 encoding for VP7. All nine AHSV serotypes were tested along with spleen samples of horses that died of AHS, serum and spleen samples from uninfected donor horses as well as non-infected *Culicoides* homogenates. Other pathogens like BTV, Equine influenza virus, vesicular stomatitis virus and WNV were tested to successfully prove specificity. The sensitivity was remarkably higher compared to previous publications with the detection limit ranging from 0.001 − 0.15 $TCID_{50}$/ml with the rRT-PCR and 0.001 − 0.8 $TCID_{50}$/ml with the conventional amplification assay. Compared to conventional RT-PCR using the primer set recommended in the OIE Manual of Standards for Diagnostic Tests and Vaccines (OIE, 2010), the sensitivity of the assays used by Fernández-Pinero et al. (2009) was increased 10^1 − 10^3-fold. In 2010, Quan et al. (2010) developed a quantitative duplex rRT-PCR (rRT-qPCR) assay optimised for the detection of AHSV in blood samples, targeting S8 encoding for VP7 and S9 encoding for NS2. By targeting two genes not related to each other, the sensitivity could be increased remarkably. The assay was unique in using circulating field isolates instead of laboratory reference strains of AHSV.

Tissue samples as well as fresh or frozen blood may be assayed – an advantage for the veterinarian in the field.

Culicoides biting midges

Biting midges of the *Culicoides* genus (Diptera: Ceratopogonidae) are haematophagous arthropods with a worldwide distribution. Approximately 1 500 *Culicoides* species have been identified worldwide (Wilson et al., 2009) of which more than 100 species occur in South Africa (Nevill et al., 1992b). In general, the most abundant species in South Africa is *Culicoides* (*Avaritia*) *imicola* Kieffer (Venter et al., 2000), followed by *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel in certain areas and depending on climatic conditions. Other commonly encountered species near livestock in South Africa are *Culicoides* (*Remmia*) *subschultzei* Cornet & Brunhes, *Culicoides magnus* Colaco, *Culicoides zuluensis* de Meillon, *Culicoides* (*Beltranmyia*) *pycnostictus* Ingram & Macfie, *Culicoides* (*Meijerehelea*) *leucostictus* Kieffer, *Culicoides* (*Beltranmyia*) *nivosus* de Meillon, *Culicoides* (*Remmia*) *schultzei* Enderlein and *Culicoides* (*Remmia*) *enderleini* Cornet & Brunhes (Meiswinkel et al., 2004).

Figure 5: *Culicoides* (*Avaritia*) *imicola* Kieffer © J. C. Delécolle

Culicoides biting midges are relatively tiny flies, measuring only 1 – 3 mm. Their grey- and white-patterned wings are used for species identification (Meiswinkel et al., 2004). They undergo four larval stages and a pupa stage before becoming an adult with one generation requiring a minimum of 25 days (Meiswinkel et al., 2004). Midges have an average life expectancy of less than 20 days, but some of them can survive up to 90 days (Mellor et al., 2000). The adult female has to take a blood meal for maturation of her eggs. Nulliparous females do not have follicular relics and have never developed eggs, parous females have got follicular relics and have laid at least one batch of eggs, and gravid females contain maturating eggs

(Braverman and Mumcuoglu, 2009). Female midges can be differentiated by Dyce's method (Dyce, 1969), which is based on the presence of pigmentation of the abdomen of parous individuals. In blood-fed, gravid and male midges, this pigment cannot be seen but they deviate morphologically and thus can be easily identified. A recent study showed Dyce's method to be inaccurate (by a factor of 23%) for determination of nulliparous and parous *C. imicola* (Braverman and Mumcuoglu, 2009). Whether it is also inaccurate for other species is currently not known. As there are no reliable alternatives for age-grading at present, Dyce's method is utilized for identification and age-grading of *Culicoides* midges whilst acknowledging its limitations.

Culicoides midges are crepuscular or nocturnal (Kettle, 1995), preferring warm, humid and calm nights (Boorman, 1993). Distribution and seasonality of the midges depend on the temperature and other climatic conditions, e.g. *C. imicola* is most abundant in late summer and autumn whereas *C. bolitinos* is most prevalent in cooler highland areas with higher rainfall (Venter and Meiswinkel, 1994). Availability of a suitable breeding habitat may be even more important. Dry soils effectively restrict the occurrence of *C. imicola*, whose pupae need damp, organically enriched soil, like most livestock associated species. *Culicoides bolitinos* will not be affected by change of soil moisture, because they breed in the dung of large herbivores, predominantly African buffalo (*Syncerus caffer*) and cattle (various *Bos* races) (Meiswinkel, 1989), and also Blue Wildebeest (*Connochaetes taurinus*), thus limiting its distribution to the ranges of these animals. Other larval habitats, only used by few *Culicoides* species, include tree-holes, plant and rock cavities as well as rotting fruits and plants (Meiswinkel et al., 2004). Most *Culicoides* species, including *C. imicola*, are not inclined to enter buildings, making stabling of animals a useful risk reduction technique for biting attacks. *Culicoides bolitinos* on the other hand has been suggested to be endophilic, i.e. preferring indoors, when large numbers were collected inside horse stables. In order to protect stabled animals from this vector, the stable needs to be adequately closed (Meiswinkel et al., 2000).

Culicoides biting midges are associated with multiple diseases important to veterinarians. Apart from being one of the causes of equine insect bite hypersensitivity (EIBH) in horses, they transmit multiple species of nematodes and protozoa (Meiswinkel et al., 2004). Of the greatest veterinary importance, however, are the viral diseases that can be transmitted by *Culicoides* species, especially diseases caused by orbiviruses, such as AHSV, BTV and EEV. Only 10 species of *Culicoides* are involved in the transmission of orbiviral diseases affecting

livestock worldwide. The most important vectors in Africa are *C. imicola* and *C. bolitinos* (Meiswinkel et al., 2004), transmitting BTV and AHSV and suspected to act as vectors for EEV (Mellor et al., 1990). Nevill et al. (1992b) investigated other *Culicoides* midges that could potentially transmit orbiviruses and identified *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro, *C. zuluensis*, *C. magnus*, *C. pycnostictus* as well as some members of the *C. schultzei* group as potential vectors. To qualify as a vector, species have to occur abundantly in an endemic area, have to prefer to feed on the animal host they are proposed to infect, and the virus must be isolated from field-caught insects. Lastly, the larval habitat has to be accordant as it also gives an indication for host association and thus helps identifying potential vectors (Nevill et al., 1992b). AHSV has been isolated from field-caught *C. imicola* as well as from *C. bolitinos* (Nevill et al., 1992a; Meiswinkel and Paweska, 2003). These two species were also proven to be susceptible to AHSV infection under laboratory conditions (Venter et al., 2000) and to feed on horses, which could be shown by blood meal identification. Other proven horse-feeders are *C. gulbenkiani*, *C. zuluensis*, *Culicoides* (*Pontoculicoides*) *engubandei* de Meillon, *Culicoides brucei* Austen, *C. schultzei* group, *C. pycnostictus* and *Culicoides* (*Avaritia*) *glabripennis* Goetghebuer (Nevill et al., 1992b). In order to investigate the oral susceptibility of *Culicoides* to live-attenuated vaccine strains of AHSV, Paweska and Venter (2003) infected 17 *Culicoides* species commonly occurring in South Africa with different serotypes. Apart from *C. imicola* and *C. bolitinos*, six other species tested positive for AHSV after 10 days of incubation: *Culicoides* (*Synhelea*) *bedfordi* Ingram & Macfie (AHSV-7), *C. magnus* (AHSV-3, -4), *C. pycnostictus* (AHSV-2), *C. zuluensis* (AHSV-2, -4), *Culicoides* (*Synhelea*) *dutoiti* de Meillon (AHSV-7) and *C. engubadei* (AHSV-4). Other species including *C. gulbenkiani*, *C. leucostictus*, *Culicoides milnei* Austen, *Culicoides* (*Remmia*) *nevilli* Cornet & Brunhes, *C. nivosus* and *Culicoides coarctatus* Clastrier & Wirth tested negative. They concluded that transmission of the vaccine strains of AHSV-4 and AHSV-7 by *Culicoides* from vaccinated to unvaccinated animals may occur (Paweska and Venter, 2003).

Culicoides imicola is a member of the *Imicola* complex, which comprises at least 12 species, of which 10 occur in Africa (Meiswinkel and Paweska, 2003) and all of which are regarded as vectors for BTV and AHSV, and potentially for EEV (Venter et al., 1999). *Culicoides imicola* has been found to feed on cattle, sheep, horses, pigs, goats and poultry, whereas only cattle, horses and sheep have been shown to be hosts for *C. bolitinos*. *Culicoides bolitinos*, also a member of the *Imicola* complex, has been involved in outbreaks of AHS in South Africa when AHSV-6 was isolated from identified pools of *C. bolitinos*. In certain regions of South

Africa it is the most abundant species, comprising up to 90% of the catches. BTV has also been isolated from identified pools of *C. bolitinos* and this species is considered a likely vector for EEV (Meiswinkel et al., 2004). BTV has additionally been isolated from *C. gulbenkiani*, but due to its limited distribution, *C. gulbenkiani* is only believed to play a minor role in the epidemiology of BT. *Culicoides magnus* may be of importance for transmission of orbiviral diseases because it occurs abundantly in parts of the African continent and belongs to the *Pulicaris* complex, a complex that is suspected to play a role in the transmission of orbiviruses (Mellor et al., 1990). The *Variipennis* complex is of importance in North America and includes *C. v. sonorensis, C. v. occidentalis* Wirth & Jones and *C. v. variipennis* (Mellor and Boorman, 1995), the first being the primary vector of BTV in North America (Tabachnick, 1996). *Culicoides* (*Hoffmania*) *insignis* Lutz extends further southwards and is the most important vector in Central America (Mellor and Boorman, 1995). In northern Europe, most common species are *Culicoides* (*Avaritia*) *obsoletus* Meigen and *Culicoides* (*Culicoides*) *pulicaris* Linnaeus (Mellor et al., 1990). However, *C. imicola* also occurs in southern Europe and played an important role in the AHS outbreak in Spain in 1987. During this outbreak AHSV was isolated from European *C. imicola* for the first time, increasing the awareness of AHS in Europe. AHSV was also isolated from mixed pools of *Culicoides* biting midges, which contained *C. pulicaris* and *C. obsoletus*, but not *C. imicola* - the first time that AHSV was isolated from *Culicoides* other than *C. imicola* (Mellor et al., 1990). The risk of these species acting as vectors for AHSV is especially high if the range of *C. imicola*, *C. pulicaris* and *C. obsoletus* overlap and virus can be transmitted between species via infected hosts. Most studies from Europe and the United States focus on *Culicoides* and the occurrence and transmission of BTV (Carpenter et al., 2009), hence sheep are mainly used as bait animals and studies concentrate on *C. imicola*, *C. obsoletus* and *C. sonorensis*. Many of the findings for BTV and its vectors are considered applicable to AHSV and its vectors, but further investigations are still needed.

AHSV in *Culicoides* biting midges

The occurrence of AHSV in field-caught *Culicoides* biting midges is of special interest, firstly because it determines the risk of infection for horses in a specific region, and secondly, it is essential for specifying a *Culicoides* species as vector for AHSV. The first time AHSV was isolated from *Culicoides* midges it was groundbreaking news that led to the conclusion that *Culicoides* biting midges are vectors for AHSV (Du Toit, 1944).

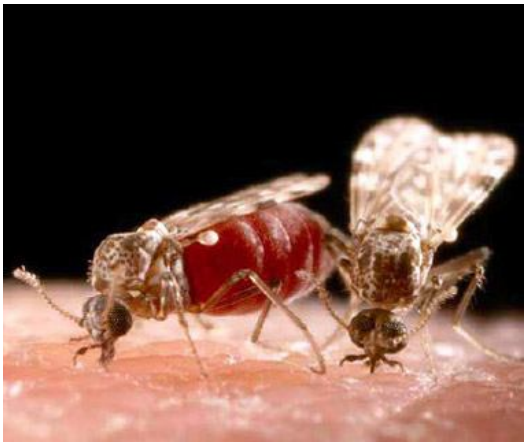

Figure 6: Blood-engorged *Culicoides* midges (published in Wilson A, Darpel K, Mellor PS (2008) Where Does Bluetongue Virus Sleep in the Winter? PLoS Biol 6(8): e210, © 2008 Wilson et al.)

Du Toit produced a case of AHS by injecting a horse with an emulsion of field-caught *Culicoides*. The infection could be reproduced in a susceptible horse by injecting blood of the first case. It was therefore anticipated that transmitters of AHSV would be found within the genus *Culicoides* (Du Toit, 1944). Between 1979 and 1985 Nevill et al. (1992a) were able to isolate three different serotypes of AHSV from *C. imicola*: Serotype 2, 4 and 7. AHSV has been isolated from field-caught *C. imicola* as well as from *C. bolitinos* (Nevill et al., 1992a; Meiswinkel and Paweska, 2003), and Venter et al. (2000) demonstrated both species to be susceptible to AHSV infection in vitro.

When aiming to detect virus in *Culicoides* biting midges, it is considered sufficient to evaluate parous or gravid individuals only (Allingham and Standfast, 1990; Venter and Meiswinkel, 1994). As there is no evidence of transovarian transmission of the virus (Jones and Foster, 1971; Nunamaker et al., 1990), males and nulliparous individuals may be excluded from such investigations (Venter and Meiswinkel, 1994). BTV has not been found in oocytes, supporting the conclusion that orbiviruses cannot be transmitted vertically and suggesting the presence of a transovarial transmission barrier (Fu et al., 1999).

Culicoides biting midges, like other arthropod vectors, have adapted their saliva to facilitate vector transmission and allow virus to be transmitted with a single bite (O'Connell, 2002). The effects, collectively termed saliva activated transmission (Randolph and Nuttall, 1994), include inhibiting host immunological response, promoting viral replication and modulating blood flow at the feeding site on the host (Wilson et al., 2009). Only a very small percentage of the adult *Culicoides* midge population will actually transmit virus, because a chain of

events have to occur in order to allow successful virus transmission. Firstly, the midge has to feed on a viraemic host. Secondly, it has to be competent to transmit virus, i.e. not have a genetic disposition for a MIB, MEB or dissemination barrier. Thirdly, it has to survive the viral EIP of about 10 days and finally, feed on a susceptible host after the end of the EIP (Wittmann and Baylis, 2000). The likelihood of one individual fulfilling all these criteria is extremely low, but this is compensated for by huge midge population sizes. Climatic conditions are very important for warm temperatures influence the population size by shortening the life and reproduction cycle and increasing activities such as mating and blood-feeding of midges. By reducing the gonotrophic cycle, midges reach the adult stage earlier and begin to feed on hosts sooner. Higher temperature also increases the biting rate (Linley, 1966; Mullens and Holbrook, 1991). Even though longevity is reduced, the EIP is shortened, enabling more midges to transmit virus. In the last century, the global mean temperature has risen by 0.5°C (Jones and Wigley, 1990) and could rise by a further 2°C by the year 2100 (Houghton, 1997). By changing the size of the adult population, climate change is therefore likely to affect vectorial capacity of *Culicoides* biting midges. A higher temperature will result in greater number of midges per year, increased chances of overwintering, increased biting rates and increased chances of surviving the EIP. It may increase the leaky-gut phenomenon turning non-vector *Culicoides* species into vectors (Mellor et al., 1998; Wittmann, 2000; Wittmann and Baylis, 2000). Adult *C. imicola* are only able to survive winter with an average daily maximum temperature of >12.5°C (Sellers and Mellor, 1993). It is believed that the northern extent of *C. imicola* in Europe has until now been limited by low temperatures (Baylis and Rawlings, 1998; Rawlings et al., 1998). Due to climate change, *C. imicola* may soon extend further north, potentially bringing BTV and AHSV into the range of the local *C. obsoletus* and *C. pulicaris* in Europe.

Fu et al. (1999) investigated barriers to BTV infection of *C. variipennis* and could not detect a salivary gland infection barrier or a salivary gland escape barrier, which have been described in other haematophagous species. In their study, midges were infected orally and via intrathoracic inoculation. The latter resulted in 100% infection rate and the virus was found in saliva of all tested individuals, both from a competent and a refractory colony. Orally infected midges were also positive for BTV right after feeding. However, only approximately 34% of the midges became persistently infected. This showed that the alimentary tract of *C. variipennis* presents a barrier to BTV infection, showing the efficacy of the MEB. Saliva of 12.1% of the orally infected midges was positive for BTV, but only out of the competent colony. Virus

titres of individuals of the refractory colony were too low to be classified as positive. The fact that the virus only disseminated in a proportion of the orally infected *Culicoides* midges strongly suggests a MEB. In a previous study, Jennings and Mellor (1987) also found that 43.6% of *C. variipennis* exhibited a MEB to BTV, restricting the virus to the gut cells. Virus was observed in the salivary glands at five days after ingestion of an infective blood meal and first transmission was observed seven days after ingestion. Fu et al. (1999) demonstrated that a minimum BTV titre in an individual of $10^{3.0}$ $TCID_{50}$ was necessary for virus transmission via saliva.

When Venter and Paweska (2007) tested a total of 3 600 parous female *C. imicola* and *C. bolitinos* collected in South Africa, none of them tested positive for AHSV, indicating a field infection prevalence of less than 0.03%, if at all present. In a six-year survey, AHSV was isolated on 66 occasions from 4 506 *Culicoides* pools, resulting in a field infection rate of less than 0.0001% (Nevill et al., 1992a). During an AHS outbreak in South Africa in 1999, AHSV-7 was isolated from one out of 334 *C. imicola* pools. During 2004, AHSV-7 was isolated once and AHSV-1 twice from 97 pools. Field infection rates of parous *C. imicola* were 0.003% and 0.002%, respectively (Venter et al., 2006). When *C. bolitinos* was the dominant species in an outbreak, its prevalence was similarly low (Meiswinkel and Paweska, 2003). After the outbreak in Spain in 1988, AHSV-4 was isolated from six out of 31 pools of non-engorged female *Culicoides*. Four of these pools consisted of *C. imicola*, but two isolations were made from mixed pools, consisting mainly of *C. pulicaris* and *C. obsoletus*. These findings are of importance since *C. pulicaris* and *C. obsoletus* extend much further north than *C. imicola* and are the most common midges in northern Europe and the United Kingdom, potentially increasing the risk of AHS reaching northern Europe (Mellor et al., 1990).

Vectorial capacity

The vectorial capacity of an insect vector is defined as the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding on a single host in one day (Black IV and Moore, 1996). It is a product of the number of vectors feeding on a host, their survival rate and the number of blood meals after their EIP. The number of vectors feeding is a product of vector density on a host and the probability of feeding each day. The probability of surviving the EIP is dependent on the vector's probability of survival each day and length of the EIP. The number of blood meals after the EIP is a product of the probability of feeding on a host and the life expectancy of the vector. The vectorial capacity can be cal-

culated by an adaption of Macdonald's equation, which was used for investigating malaria in Africa:

$V = m\, a^2\, b\, p^n / (-\ln p)$.

V = number of new infections arising per day from a currently infective case = vectorial capacity
m = vector density on host
a = probability of blood meals taken by a vector per host per day
b = proportion of vectors ingesting an infective meal and successfully becoming infective = vector competence
p = probability of vector's daily survival rate
n = duration of EIP
$1/(-\ln p)$ = duration of vector's life after surviving EIP

Of all the variables a, n, and p affect the equation the most; especially the daily survival rate p. The daily survival rate can be effectively reduced by adulticides, making them a convenient tool in order to reduce the vectorial capacity of *Culicoides* biting midges.

Trapping methods for *Culicoides* biting midges

A variety of methods are available for catching insects. It has been reported that *Anopheles* species are attracted to a host by carbon-dioxide (CO_2) (Dekker and Takken, 1998), heat, moisture and specific odours, which are elements some trapping devices revert to. Another well known attractor for insects is ultraviolet (UV) light, utilized by light traps, the primary monitoring tool all over the world. White light as well as blacklight traps are available, the latter being more attractive to insects and, hence preferred by researchers. Insects attracted towards the UV light are blown into a collection beaker by a fan (usually creating a downdraught). Advantages of a blacklight trap are the large number of insects that can be collected, its user friendliness because it does not need to be frequently monitored, and its ability to collect dead and live midges. Collected midges are suitable for virus isolation, blood meal identification, age-grading and taxonomic studies. Disadvantages are the necessity for electricity which can limit collections in rural areas and the required journey to a disease outbreak. Battery operated light traps are available, but the light source is weak due to the low power supply and attracts less insects than light traps operated with electricity. Additionally,

the suction fan may damage *Culicoides* midges and collections can get extremely 'dirty' because of contamination with other larger insects. Larger insects may be excluded by a mesh fastened around the apparatus. Although very effective and widely used, blacklight traps are considered biased samplers and may not be suitable for specialized studies (Meiswinkel et al., 2004).

Figure 7: Onderstepoort light trap. © Elli Scheffer

One of the first scientists to document usage of a light trap for trapping *Culicoides* biting midges was DuToit (1944). Subsequently, light traps have been used to collect insects in multiple studies and have also been subject to comparative studies (Venter et al. 2009a; 2009b). These studies discussed different light trap types and the effect of light trap height on collections of *Culicoides* species in South Africa. Comparing five different light traps used in Europe, Africa and North America, namely the Onderstpoort trap, the Rieb trap, the Miniature Centre for Disease Control (CDC) light trap, the Pirbright trap and the BG-sentinel mosquito trap, the Onderstepoort downdraught suction light trap was concluded to be the most effective for *Culicoides* collections and the most sensitive in collecting rare *Culicoides* midge species. With a 30 cm 8 W UV light tube it contains the strongest light source, which could explain the significantly higher number of *Culicoides* midges collected with this trap. The polyester

netting supplied with the Onderstepoort light trap has a mesh size of only 2 mm compared to 5 mm nettings used by the other light traps, causing the ratio of non-*Culicoides* and *Culicoides* to be most favourable in the Onderstepoort light trap.

Disadvantages of light traps are that they become less efficient as wind speed increases and that they do not accurately reflect midge biting rates (Taylor, 1962; Meiswinkel et al., 2000). While they give an overview of the midge population in an area, mechanical aspiration off bait animals is a more accurate indicator of the midge biting rates on the hosts. This is of special interest in determining the risk of pathogen transmission by insects (Gerry et al., 2008). A manual aspirator, where insects are sucked into a tube by means of a glass mouthpiece, was not successful when used in 1944 by Du Toit to catch potential AHS vectors (Du Toit, 1944). Modern devices are electrical and have been used as early as 1974 with the aim to determine the cause of EIBH, also known as sweet itch (Mellor and McCraig, 1974). Using eight bait horses in England, investigators collected a total of 1 911 *Culicoides* of seven different species. The predominant species was *C. obsoletus*, which clearly favoured the ventral midline of the horses, as did most of the other species. *Culicoides pulicaris*, which they concluded to be the most probable cause of sweet itch, preferred the withers. Jones et al. (1977) collected a total of 1 339 *C. variipennis* with a mouth aspirator and a battery operated aspirator in the United States, alternating 5 minute collection with 5 − 10 minute non-collection periods over sunrise and sunset. Results showed a preference of *C. variipennis* to attack the bait horse during the morning hours, and on the ventral midline. Difficulties were encountered with visualizing the small insects at dusk and by the bait animals being startled by the battery-operated aspirator. During the next decade, studies on horses were performed in New York, USA (Schmidtmann et al., 1980), Ireland (Townley et al., 1984) and Israel (Braverman, 1988). The former study used a tethered cow, sheep and horse as bait animals which were sampled for a 1 minute period at 30 minutes before, 10 minutes before and 10 minutes after sunset. Four regions on the belly, brisket, back and neck were brushed vigorously before each sampling and thus the investigators managed to collect four *Culicoides* species off the horse, mostly from the belly region: *Culicoides* (*Silvaticulicoides*) *biguttatus* Coquillett, *C. obsoletus*, *Culicoides* (*Oecacta*) *stellifer* Coquillett and *Culicoides* (*Silvaticulicoides*) *spinosus* Root & Hoffmann (Schmidtmann et al., 1980). Townley et al. (1984) investigated preferred landing sites of *Culicoides* midges and divided the horse's body into 11 regions, each of which was sampled with a portable car vacuum cleaner for 10 minutes between 8 pm and 10 pm. Over 15 evenings, they managed to collect 10 282 *Culicoides* midges, with *C. obsoletus*

and *Culicoides* (*Avaritia*) *dewulfi* Goetghebuer each comprising about 45% of the total. These were found mostly on the hind quarters and then decreasing on the mane, lower legs, middle thorax/abdomen and neck. It is possible that the presence of long hair on the lower limbs of the draught-type bait horse increased attraction to that specific area (Townley et al., 1984). Braverman collected a total of 620 *Culicoides* midges of five different species, mostly *Culicoides* (*Monoculicoides*) *puncticollis* Becker and *C. imicola*, from a tethered horse in Israel. Collections were performed 60 minutes prior to 30 minutes post sunset with 20 minute collection periods followed by 10 minutes of non-collection, using a modified car vacuum cleaner. Preferred landing sites were evaluated, and except for two species, which were found almost exclusively on the belly region (99% of *C. puncticollis* and 95% of *C. schultzei* group), *Culicoides* seemed to prefer the dorsal parts of the animals from the mane to the tail base (Braverman, 1988). This is the only study investigating the preferential landing behaviour of *C. imicola*. Seventeen percent of *C. imicola* were found on the mane and withers, 45% on the upper back, 9% on rump and tail base, 2% on the shoulder and 28% on the belly region. Greiner et al. (1990) aspirated a total of 2 933 female *Culicoides* of nine species from a horse when investigating the cause of insect hypersensitivity in Florida, U.S.A. Aspirating from 60 − 90 minutes before to 60 − 90 minutes after sunset and repeating the same time pattern at sunrise, three to four collections per hour were performed. A light source was not used for collections in the dark and nine regions on the horse's body were sampled. *Culicoides insignis* comprised 90% of the aspirated midges. This species along with four other species preferred the ventral belly region, while *Culicoides* (*Avaritia*) *alachua* Jamnback & Wirth and *Culicoides* (*Avaritia*) *pusillus* Lutz preferred the dorsal portions. The flank was the least frequently 'attacked' site (Greiner et al., 1990).

Eight years later, Mullens and Gerry (1998) compared collections off a bait calf using a drop trap to those using CO_2 suction traps. A drop trap is lowered around a bait animal that has been exposed for a certain period of time. Potential vector insects can be aspirated from the mesh of the drop trap with a portable vacuum aspirator (Fletcher et al., 1988; Carpenter et al., 2008; Mullens et al., 2010). In a CO_2-baited trap, insects are attracted by CO_2 instead of UV light. The advantage of this trapping device is that it only attracts insects in search of a blood meal, whereas light traps attract a broader range of insects (Mullens et al., 2005). Mullens and Gerry (1998) reported sampling at 30 minutes intervals, consisting of 10 minutes exposure time, 10 minutes for lowering the net and allowing the insects to leave the host and 10 minutes of collection, with the investigator vacuuming the interior of the net. A total of 3 709

C. sonorensis were collected 30 minutes before sunset, at sunset, 30 and 60 minutes after sunset, with up to 281 engorged *Culicoides* collected in a single 10 minute exposure period. Even though the collections from the calf and the CO_2 trap were highly correlated ($r = 0.82$, $P < 0.01$), significantly more *Culicoides* midges were collected from the calf, showing that even though CO_2 is an effective attractant, other host cues or the manner of release (pulsed in exhaled air vs. stable in baited trap) is of importance. A correction factor when using CO_2-baited traps was proposed (Mullens and Gerry, 1998). Investigating equine dermatitis, Mullens et al. (2005) sampled 61 *Culicoides*, comprising only *C. obsoletus* and *C. sonorensis*, on 11 collection dates from the ventral midline of a horse, the preferred feeding site of *C. sonorensis*. Collections were made with a vacuum aspirator 1 hour before and 1 hour after dusk. Results were compared to CDC-type suction traps and CO_2-baited traps, which collected *Culicoides* more effectively, but underrepresented *C. obsoletus* (Mullens et al., 2005). Gerry et al. (2009) compared *Culicoides* biting midge occurrence on a tethered sheep in Spain using a modified car vacuum cleaner to results obtained in a CDC-type suction trap and a CO_2 suction trap. The trapping devices were assigned randomly. Sampling took place 90 minutes before until 90 minutes after sunset with a 5 minute collection period followed by 15 minutes of non-collection for the suction trap, whilst the UV light and CO_2-baited traps ran throughout the entire 3 hour trap period. A total of 709 *Culicoides* midges of five species, mainly *C. parroti* and *C. obsoletus*, were aspirated, whereas the other trapping devices collected 783 and 41 *Culicoides*, respectively. Significant differences between the investigated trapping methods in regards to species distribution were noted: the UV light traps underestimated the sheep biting rate of *C. obsoletus* and overestimated that of *C. imicola.* Gerry et al. (2008) also compared different trapping methods using a mechanical aspirator on a horse and a CDC-type CO_2 suction trap. A total of 3 056 *C. sonorensis* were collected with both trapping devices on 21 collection days in California, U.S.A. Both traps ran simultaneously from 7 pm to 12 am with a 45 minute collection period following 15 minutes of recovery. Investigators were equipped with headlamps to locate insects after sunset and additionally vacuumed the ventral midline of the horse without visualizing the insects in this area. Their results showed the highest biting rate just after sunset, with biting midges favouring the ventral midline. Due to expected underrepresentation of *Culicoides* midges using the aspirator, no further tests were performed to compare results to the CO_2 suction trap (Gerry et al., 2008). To date no mechanical aspirations of *Culicoides* off hosts have been described in sub-Saharan Africa.

Figure 8: Mechanical aspirator. © Elli Scheffer

Mechanical aspiration allows investigation of midge numbers on a single animal and even a finely specified area on that animal. Furthermore, the collected insects generally remain intact. Disadvantages of mechanical aspiration include the influence of weather conditions, labour intensity (van der Rijt et al., 2008) and possibly modified insect behaviour due to a restrained animal (Mullens and Gerry, 1998). Whilst a human investigator may be considered to influence results experiments conducted with and without animals but with consistently present humans showed that the presence of humans has a negligible effect. Humans attract few or no *Culicoides* compared to horses, probably because humans produce less CO_2, heat and odours than horses (van der Rijt et al., 2008). One study (Mullens and Gerry, 1998) reported that even though numbers of *Culicoides* aspirated from three bait calves were similar, the engorgement rate varied significantly, favouring one of the bait calves. Van der Rijt (2008) also observed that *Culicoides* biting midges showed marked differences in attraction to individual bait animals. This is probably due to semiochemicals, which emanate from body, breath and excrements of vertebrate hosts and which can influence the physiological behaviour of midges, such as mating, breeding and host-seeking. Haematophagous insects have a highly developed olfactory system and detect substances like CO_2 or 1-octen-3-ol with their antennae. These semiochemicals can function as attractants and can, along with pheromones of *Culicoides*, be utilised as bait for trapping devices. Other semiochemicals can act as repellents. It is likely that production of repelling semiochemicals is genetically determined in animals, suggesting breeding programs to decrease potential risk for farm animals. The most promising method to reduce the risk of animals being bitten seems to be a so-called push-pull

control strategy, where a repellent semiochemical is either used on or around the animal, or a genetically repelling animal has been bred, and a trap baited with an attractant is simultaneously used to lure potential vectors away from the animals (Logan and Birkett, 2007).

Results of collections of *Culicoides* midges obtained with a light trap and with mechanical aspiration are not directly comparable. Jones et al. (1977) already stated in 1977 that total numbers cannot be compared, because aspiration is dependent on the efficiency of the investigator and light traps do not work as well during morning crepuscular hours. In addition, as different attractants are used – light vs. the host animal – different results are to be expected. In their study, Gerry et al. (2009) concluded it vital to conduct animal-based collections along with UV light and CO_2-baited trap collections in order to interpret the epidemiological significance of the trap collections. Mullens and Gerry (1998) could show a correlation between aspirated *Culicoides* midges and collections in a CO_2-baited trap. However, total numbers were significantly different with the aspirator catching 3.4 – 6.1 times more midges. When assessing surveillance techniques for *Culicoides* biting midges in Europe, Carpenter et al. (2008) concluded that light trapping surveillance does not provide an accurate reflection of biting populations of *Culicoides* on sheep used as bait animals.

Apart from UV light traps and mechanical aspiration, other trapping devices have been described. The Counterflow geometry (CFG) trap can use various baits such as pheromones or host odours like 1-octen-3-ol and CO_2. Originally sold as the Mosquito Magnet®, it has been described to also be effective for *Culicoides* biting midges (Logan and Birkett, 2007). Vehicle-mounted traps use a large net in which insects are captured by the movement of the vehicle. These traps are particularly useful for monitoring *Culicoides* midges during the day, collecting male swarms or collecting near wildlife. Collected specimens can be used for taxonomic studies. Along with non-attractive suction traps vehicle mounted traps are considered the least biased trapping devices. Emergence traps are installed on top of a suspected larval habitat and catch newly developed *Culicoides* biting midges. Larvae and pupae can also be retrieved with this method, which is helpful for investigating larval habitats and requirements for the immature stages of *Culicoides* species (Meiswinkel et al., 2004).

Trapping is most successful during the time of day that *Culicoides* biting midges are most active. Since most *Culicoides* species are crepuscular and/or nocturnal (Braverman, 1994), trapping should preferably be performed around sunset. Van der Rijt (2008) used a drop trap

to collect *Culicoides* midges at sunrise, in the early afternoon, at sunset and at night, catching clearly the largest amount at sunset. Braverman (1988) was only able to collect *Culicoides* midges at sunset when he used a suction trap at hourly intervals. Jones et al. (1977) on the other hand found a larger number of *Culicoides* during the early morning hours. However, vision sufficiency at dusk was queried and the collected *Culicoides* midges were only comprised of *C. variipennis*, whose peak of activity might differ from that of other *Culicoides* species. The same applies to *Culicoides* (*Culicoides*) *impunctatus* Goetghebuer, which has been mostly collected during morning crepuscular hours (Blackwell, 1997).

Culicoides and climate variables

Meteorological factors influence not only the distribution and vectorial capacity of *Culicoides* biting midges, but also their occurrence and the number of collected insects. Wind speed has a negative influence on midge collections with windy evenings decreasing midge host seeking activities. Braverman (1988) named wind as the most influential meteorological factor for collecting *Culicoides* biting midges. Wind also influences *Culicoides* distribution by causing passive dispersal of vectors for distances of up to 700 km. Meiswinkel et al. (2000) confirmed previous findings when they showed that catches of *C. imicola* and *C. bolitinos* are positively related to temperature, negatively to humidity and negatively to wind speed in outside collections. Similar findings were reported for *Culicoides* species in Kenya (Walker, 1977). Blackwell (1997) also confirmed a positive relationship for temperature and catches of *C. impunctatus,* but found catches to also correlate positively with relative humidity. In a study conducted in the United Kingdom, Carpenter et al. (2008) measured air temperature, humidity, dew point, mean and maximum wind speed, wind direction and variation in direction as well as solar radiation. Out of these variables, air temperature and wind direction significantly influenced the number of blood-fed midges collected, optimum air temperature being 20 − 22ºC. If temperature rose above 25ºC or fell below 15ºC, the biting rate decreased. Ortega et al. (1999) showed a prerequisite air temperature of 18 − 38°C for *C. imicola* activity in Spain. High solar intensity (>200 Wm^{-2}) and high humidity, both of which are related to high temperatures, also decrease biting midge activity, as do high maximum wind speeds (>3 ms^{-1}) and a high degree in wind turbulence (>40º variation) (Carpenter et al., 2008). Nevill (1967) discovered that over 700 mm of rain/autumn are not tolerated by *C. imicola* pupae. Adult midge numbers, however, increase after heavy rainfall (Meiswinkel, 1998) and above average rainfall can be linked to outbreaks of *Culicoides*-borne orbiviral diseases (Meiswinkel et al., 2004). Looking at previous epizootics in South Africa, a strong association has been found

between the epizootics and the warm phase of the El Niño/Southern Oscillation (ENSO). ENSO has been correlated with epidemics of several mosquito-borne diseases and, likewise, 13 out of 14 major AHS epizootics in South Africa coincided with the El Niño phenomenon which is typically heavy rain followed by drought. In the years of AHS outbreaks, however, drought is followed by heavy rainfall. The reason for this correlation is not yet completely understood, but it is suspected that breeding sites of the vector are altered, reservoir hosts such as zebra congregate at water holes which facilitates transmission, and that the heat wave increases vector population growth rate and virus transmission (Baylis et al., 1999).

Aims of this study

The differences between various trapping methods for *Culicoides* biting midges have been described. However, no data is available on mechanical aspiration in southern Africa, which is why this constitutes a major focus in this study. Mechanical aspiration was performed on horses with special regards to AHS in order to confirm known vectors and identify new vectors. It is anticipated to develop a practical and convenient technique for epidemiological surveys.

RT-qPCR assays, which are well described as a diagnostic tool for AHSV infection of horses, are believed to be applicable to *Culicoides* midges as well. It was the aim of this study to confirm this hypothesis and find an easy-to-perform and repeatable PCR assay capable of locating AHSV in individual *Culicoides* midges and of investigating the infection prevalence of a *Culicoides* midge population.

Barriers to replication and transmission of orbiviruses have only been investigated for BTV in *C. variipennis*. The present study has therefore investigated possibly similar mechanisms in *C. imicola*, the main vector for AHSV.

Chapter Two: Research papers

Comparison of two trapping methods for Culicoides biting midges and determination of African horse sickness virus prevalence in midge populations at Onderstepoort, South Africa

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Abstract

Culicoides biting midges (Diptera: Ceratopogonidae) are vectors of a variety of pathogens including African horse sickness virus (AHSV), a member of the family *Reoviridae*, genus *Orbivirus*. AHSV causes African horse sickness (AHS), an endemic disease of equids with an extremely high mortality rate in horses in sub-Saharan Africa. *Culicoides* (*Avaritia*) *imicola*

Kieffer is considered to be the principal vector of AHSV and is the dominant *Culicoides* species in South Africa. Due to the global distribution of *Culicoides* vectors, there is a potential risk of AHS spreading from endemic areas to areas traditionally free of the disease, which could have a severe economical impact on the affected equine industry. As part of any risk assessment it is essential to monitor known vectors as well as potential vector species. In the present study, sampling of *Culicoides* insects was compared using overnight collections in the conventional Onderstepoort light trap and mechanical aspiration of midges at sunset from bait horses. *Culicoides imicola* was confirmed as the predominant species using both trapping methods. Other species, mainly *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel and *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro, were highly underrepresented in the light trap collections, but made a significant contribution to the mechanical aspiration catches. The time for optimal collection differed between the trapping methods, leading to the conclusion that mechanical aspiration is a useful addition to conventional light trap collection and possibly the better choice when investigating insect vectors. An infection rate of 1.14% was calculated for the midge population based on real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assays of collected *Culicoides* midges, which exceeds previous estimates. This is probably due to the increased sensitivity of the RT-qPCR assay used in this study as compared to the virus isolation assays used in previous studies. RT-qPCR-positive midges were present in midge pools obtained from both light trap and mechanical aspiration. Seven of the positive pools consisted of *C. imicola* only, four contained mixed species and one pool contained no *C. imicola*, suggesting the presence of AHSV in midges of other species.

Keywords

Culicoides, collection methods, light trap, mechanical aspiration, African horse sickness virus

1. Introduction

Biting midges of the *Culicoides* genus (Diptera: Ceratopogonidae) are haematophagous arthropods, which can serve as vectors for Orbiviruses such as bluetongue virus (BTV), equine encephalosis virus (EEV) and African horse sickness virus (AHSV). *Culicoides* (*Avaritia*) *imicola* Kieffer is the most abundant species in the region surrounding Onderstepoort, South Africa, (Venter et al., 2009b) and is considered the most important vector of Orbiviruses in Africa, followed by *Culicoides* (*Avaritia*) *bolitinos* Meiswinkel (Meiswinkel et al., 2004). AHSV has been isolated from field-caught midges of both species (Nevill et al., 1992a; Meiswinkel and Paweska, 2003) and both species are susceptible to AHSV infection

under laboratory conditions (Venter et al., 2000). Light traps are commonly used to collect *Culicoides* midges and the Onderstepoort light trap is one of the most effective, as well as the most sensitive, in catching less abundant species (Venter et al., 2009b). Light traps only provide an indication of the midge population in an area and this may be biased by factors including the time of the year and the type of trap. In contrast, mechanical aspiration directly from animals provides a more accurate representation of the midge biting rate (Carpenter, et al., 2008). Although aspiration devices were evaluated as early as 1944 (Du Toit, 1944), successful utilization of this approach for midges on horses was first reported in 1974 in England (Mellor and McCraig, 1974), followed by reports from the USA (Schmidtmann et al., 1980) and Israel (Braverman, 1988). Subsequent studies have confirmed that different results for *Culicoides* occurrence are obtained using an aspirator as compared to a light trap and/or CO_2 suction trap (Gerry et al., 2008; Gerry et al., 2009).

AHSV is the cause of African horse sickness (AHS), an infectious non-contagious disease of equids that is endemic in sub-Saharan Africa. AHSV is a nonenveloped virus with a genome of 10 segments of double-stranded RNA that encodes seven structural and four non-structural proteins (Roy et al., 1994). A duplex real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) has been described recently to diagnose AHSV infection in horses, using either blood or tissue samples (Quan et al., 2010). Nevill et al. (1992a) documented an AHSV field infection rate of less than 0.0001% in a *Culicoides* population over a six year period. Even in outbreak situations of AHSV infection in 1999 and 2004 in the Western Cape Province of South Africa, field infection rates in the dominant species, *C. imicola*, were found to be only 0.003% and 0.002%, respectively (Venter et al., 2006).

The majority of prior studies involving *Culicoides* biting midges have focussed on the occurrence and transmission of BTV (Carpenter et al., 2009). Studies of BTV infection of sheep have concentrated on *C. imicola*, *Culicoides* (*Avaritia*) *obsoletus* Meigen and *Culicoides* (*Monoculicoides*) *sonorensis* Wirth & Jones, whereas there are few publications directly addressing the role of *C. imicola* in transmission of AHSV, despite its putative role as the major vector for this virus (Meiswinkel et al., 2004). Mechanical aspiration from bait horses has mostly been used to investigate summer seasonal recurrent dermatitis, also known as 'sweet itch' or equine insect bite hypersensitivity (EIBH), a hypersensitivity reaction caused by *Culicoides* biting midges (Mellor and McCraig, 1974; Townley et al., 1984; Mullens et al., 2005). However, no mechanical aspiration of horses has yet been reported in the AHSV enzootic

region of sub-Saharan Africa, in fact virtually no animal bait-trapping of *Culicoides* has been reported previously in Africa (Meiswinkel et al., 2004; Gerry et al., 2009). Furthermore the prevalence of AHSV in a *Culicoides* population in an AHS endemic area has not yet been determined in a quantitative fashion.

The objective of this study was to evaluate the relative importance of different *Culicoides* species that can potentially serve as vectors of AHSV in an enzootic area of South Africa. The light trap, a widely used surveillance tool for *Culicoides* midges, was compared to mechanical aspiration from horses and the prevalence of AHSV in the *Culicoides* biting midge population at Onderstepoort, South Africa, an area endemic to AHS, was determined by real-time RT-qPCR.

2. Materials and Methods

A field study was conducted from 10 February to 18 August 2010 at the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, Onderstepoort, South Africa (25º 38' 52" S, 28º 10' 54" E, 1,238 m above sea level). Samples were collected on three nights per week (weather permitting) during the peak vector season (February to May). With the onset of cooler weather and reduced midge numbers in June, collections were reduced to once per week.

Five healthy horses were selected, one of which served as a bait horse throughout the study. A second horse was randomly selected from the remaining horses and served as an additional bait horse on each collection day. The horses were kept together on a grass paddock (58 x 71 m). All horses had been previously vaccinated against AHS with a commercially available live-attenuated vaccine (African horsesickness Vaccine, Onderstepoort Biological Products, South Africa).

A single 220 V Onderstepoort downdraft suction light trap (ARC-Institute for Agricultural Engineering, South Africa) operating with an 8 W UV-light tube was used for insect collection, as described previously (Venter et al., 2009b). The light trap was installed underneath a roofed area of the OVAH, 1.8 m above the ground and approximately 15 m from the paddock enclosing the trial horses. Insects entering the light trap were collected in a 500 ml plastic collection beaker containing 200 ml of water and 0.2 ml of Savlon (containing Clorhexidine gluconate 0.3 g/100ml and Cetrimide 3.0 g/100ml) (Johnson&Johnson, South Africa). Insects

collected were sorted and *Culicoides* midges stored in 70% ethanol. The light trap was operated simultaneously with mechanical aspiration from 20 min prior to sunset until 5 min thereafter. The collection beaker was exchanged and a second beaker collected insects overnight, from 5 min after sunset until 2 h after sunrise the following morning. No other trapping devices were operated in the immediate vicinity of the horse paddock and the light trap.

A customized mechanical aspirator based on a 6 V Black & Decker rechargeable hand-held vacuum cleaner (2820A AC Insect vacuum, BioQuip Products Inc., U.S.A.) was used. A removable collection tube was installed, covered with fine mesh on one side and an elastic opening on the other side. The collection tube was covered with a lid as soon as it was extracted from the vacuum cleaner after aspiration, and the aspirated insects were immediately immobilized by placing the tube in a portable cooler box. A separate plastic tube was used for each horse and collected *Culicoides* insects were stored in 70% ethanol. The horses were inspected for 5 min each and visualized insects that resembled *Culicoides* midges were manually aspirated. The horses were consistently evaluated from cranial to caudal with emphasis on the dorsal regions of the neck, back and rump, which have been described as preferred landing sites of *C. imicola* (Braverman, 1988). The collection period lasted from 15 min before until 5 min after sunset, the period when *Culicoides* midges appeared to most actively feed on the horses. This allowed for three collection periods of 5 min each.

Total numbers of *Culicoides* midges were determined under a stereomicroscope and the insects were segregated according to species and parity status. Parity sorting helped increase efficiency of testing for virus presence by avoiding testing unfed, nulliparous individuals in larger collections. Males as well as nulliparous, parous, gravid and blood-fed females were differentiated using the method described by Dyce (1969). Large light trap collections were sub-sampled with the method of Van Ark and Meiswinkel (1992).

As preliminary studies had demonstrated that the RT-qPCR used in this study could detect a single infected midge in a pool of 200 *Culicoides* midges, pool size was limited to 200. Blood-fed female *Culicoides* were removed from all catches and excluded from the pools. On days where the total catch was less than 200 *Culicoides* midges, all midges were combined in a single pool regardless of their parity status as the inclusion of nulliparous females and or males would not affect the outcome of RT-qPCR testing. On days that the total catch was in excess of 200 midges, nulliparous females and males were removed and pools of up to 200

parous and gravid *Culicoides* were made from each sampling, with each pool containing only *C. imicola*, a mixture of other *Culicoides* species, or a mixture of all *Culicoides* species, including *C. imicola*. Daily catches of less than 10 *Culicoides* midges were not analysed. The pools of *Culicoides* biting midges collected throughout the six month study period were subject to real-time RT-qPCR to detect AHSV. Preparation, extraction and amplification followed an adaptation of the protocol as described by Quan et al. (2010). Briefly, 500 µl of lysis binding solution (AM8500) from the Ambion total nucleic acid extraction kit (AM1836) was added to each midge pool. The pools were homogenized in a MagnaLyser (Roche Products, South Africa), followed by centrifugation to remove the detritus. The supernatant was used for RNA extraction in the Kingfisher Automated Purification System (ThermoFisher Scientific, Finland) using the Ambion total nucleic acid extraction kit according to the manufacturer's instructions. A 5 µl volume of the RNA extract was transferred to thin-walled PCR tubes and 5 µl of the primer-probe-mix (Quan et al., 2010) added to each extract. The RNA was denatured in the presence of the primers and probes for 1 min at 95°C followed by snap cooling. Thereafter, 15 µl of master mix was added to each well and the one step RT-PCR was performed as described previously (Quan et al., 2010) using the StepOne Plus Real Time PCR System (Applied Biosystems, U.S.A.). This RT-qPCR assay detects highly conserved regions of the S8 and S9 genes of AHSV, which encode the VP7 structural protein and NS2 non-structural protein of AHSV, respectively. Negative and positive controls were included with each set of samples.

Statistical tests used in this study included Fischer's exact test, R^2 and χ^2. Infection prevalence was calculated with PooledInfRate (Biggerstaff, 2006). A value of $P \leq 0.05$ was considered significant.

3. Results

The light trap was always operated for the 20 min period during which mechanical aspiration was performed. After changing of the collection beaker, the light trap was then operated for the rest of the night (approximately 14 h). Over 42 collection nights, the light trap collected only 7 midges during the initial sampling period whereas 2 454 midges were collected by mechanical aspiration directly from horses (Table 1). A total of 49 485 *Culicoides* midges were collected during the overnight trapping period with the light trap.

	LIGHT TRAP					MECHANICAL ASPIRATION				
	number of *Culicoides*	positive catches	average per 42 catches	% of total *Culicoides*	**RANK**	number of *Culicoides*	positive catches	average per 42 catches	% of total *Culicoides*	**RANK**
C. imicola	46406	39	1105	93.8	**1**	1454	32	35	59.3	**1**
C. bolitinos	767	29	18	1.5	**2**	685	24	16	27.9	**2**
C. nivosus	418	25	10	0.8	**3**	0	0	0	0	**-**
C. leucostictus	379	30	9	0.8	**4**	1	1	<1	0	**10**
C. pycnostictus	278	24	7	0.6	**5**	0	0	0	0	**-**
C. gulbenkiani	241	22	6	0.5	**6**	247	25	6	10.1	**3**
C. bedfordi	178	19	4	0.4	**7**	0	0	0	0	**-**
C. milnei	154	14	4	0.3	**8**	7	3	<1	0.3	**7**
C. enderleini	152	15	4	0.3	**9**	1	1	<1	0	**11**
C. zuluensis	152	17	4	0.3	**10**	11	9	<1	0.4	**6**
C. coarctatus	106	8	3	0.2	**11**	0	0	0	0	**-**
C. #54 df	58	12	1	0.1	**12**	0	0	0	0	**-**
C. neavei	45	11	1	0.1	**13**	0	0	0	0	**-**
C. magnus	35	17	1	0.1	**14**	22	9	1	0.9	**4**
C. nevilli	32	6	1	0.1	**15**	0	0	0	0	**-**
C. similis	18	9	<1	0	**16**	0	0	0	0	**-**
C. expectator	17	5	<1	0	**17**	0	0	0	0	**-**
C. #50	15	7	<1	0	**18**	2	2	<1	0.1	**8**
C. glabripennis	9	3	<1	0	**19**	2	2	<1	0.1	**9**
C. #107	7	3	<1	0	**20**	0	0	0	0	**-**
C. brucei	6	2	<1	0	**21**	1	1	<1	0	**12**
C. trifasciellus	3	2	<1	0	**22**	0	0	0	0	**-**
C. subschultzei	3	1	<1	0	**23**	0	0	0	0	**-**
C. dekeyseri	2	1	<1	0	**24**	0	0	0	0	**-**
C. ravus	2	1	<1	0	**25**	0	0	0	0	**-**
Nigripennis group	1	1	<1	0	**26**	0	0	0	0	**-**
C. tropicalis	1	1	<1	0	**27**	0	0	0	0	**-**
C. tuttifrutti	0	0	0	0	**-**	1	1	<1	0	**13**
Unidentifiable	0	0	0	0	**-**	20	4	1	0.8	**5**
TOTAL	49485	42	1178			2454	42	58		

Table 1: Comparison of *Culicoides* biting midge species caught overnight by light trap and by mechanical aspiration near sunset from horses from 10 Feb, 2010 to 18 Aug, 2010 at Onderstepoort, South Africa.

Twenty-seven different species of *Culicoides* midges were caught in the light trap overnight, whereas only 12 were collected by mechanical aspiration (Table 1). Figures 9 – 12 show the total numbers of *Culicoides* midges collected throughout the six month study period. *Culicoides imicola* was the predominant species collected by both methods, with *C. bolitinos* the second most abundant (Table 1). However, the ratio of *C. imicola* and *C. bolitinos* to other

species varied significantly with collection method. Specifically, 94% of midges collected in the light trap were *C. imicola*, whereas this species only comprised 59% of the mechanically aspirated midges. Only 1.5% of the midges harvested in the light trap were *C. bolitinos* as compared to 28% of those collected by aspiration. *Culicoides imicola* was detected by mechanical aspiration throughout the study period (Fig. 9), whereas in the light trap this species was collected predominantly during summer until May. Interestingly, 10% of the midges caught by mechanical aspiration were *Culicoides* (*Avaritia*) *gulbenkiani* Caeiro whereas this species comprised only 0.5% of midges caught in the light trap. *Culicoides bolitinos* (Fig. 10) and *C. gulbenkiani* (Fig. 11), which wre both collected in negligible numbers in the light trap, showed a definite peak at the end of May by mechanical aspiration. A single *Culicoides tutti-frutti* Meiswinkel, Cornet and Dyce was the only species that was collected with mechanical aspiration, but not with the light trap. Remaining species were represented with considerably higher numbers in the light trap (Fig. 12), probably due to larger total numbers of *Culicoides* midges. A total of 20 *Culicoides* were too damaged to be identified to species level following mechanical aspiration.

Regardless of the trapping method, collections consisted mainly of non-blood-fed nulliparous and parous females, i.e. females actively looking for a blood meal (light trap 54% and 35%, mechanical aspiration 64% and 30%). Gravid females as well as males (7% and 4% respectively) were detected in the light trap, but not by aspiration. Blood-fed *Culicoides* were more common in the mechanical aspiration trap (5%) as compared to the light trap (1%). The overall distribution was similar to that previously reported for light trap collections (Venter et al., 2009b).

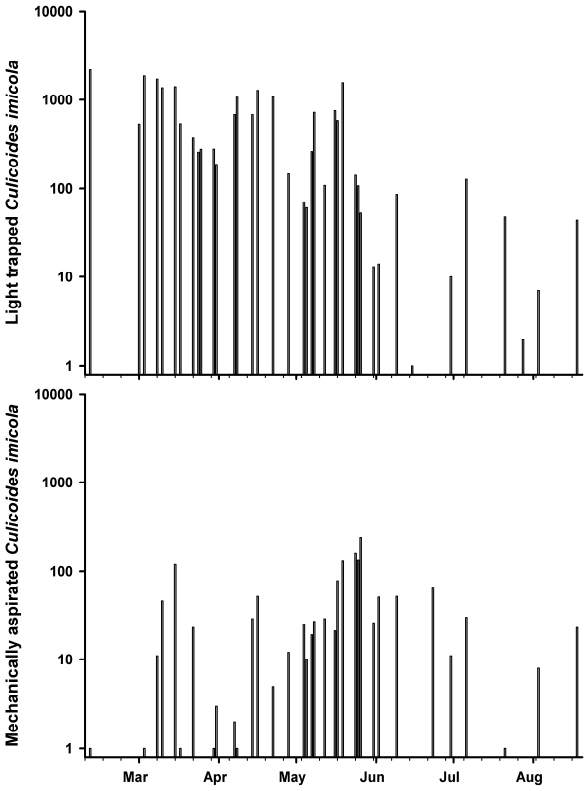

Figure 9: Number of *Culicoides imicola* collected in light trap in 14 hours and by mechanical aspiration from horses in 20 min between 10 Feb 2010 and 18 Aug 2010.

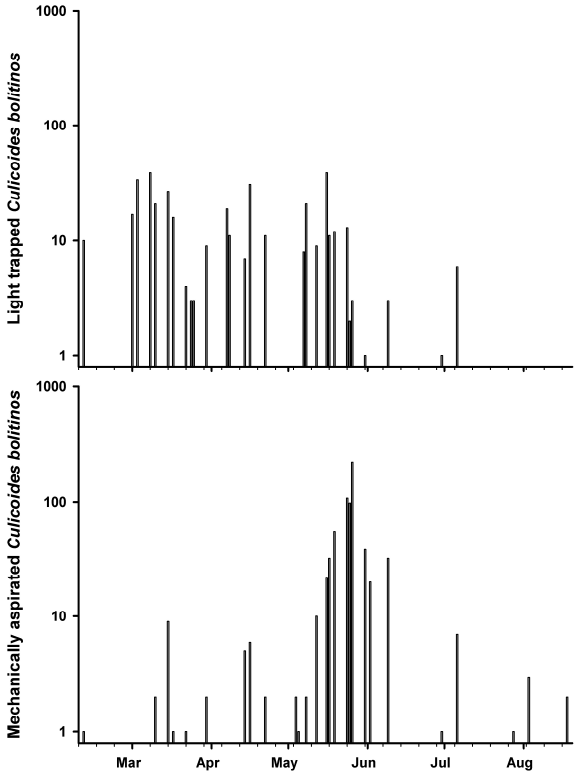

Figure 10: Number of *Culicoides bolitinos* collected in light trap in 14 hours and by mechanical aspiration from horses in 20 min between 10 Feb 2010 and 18 Aug 2010.

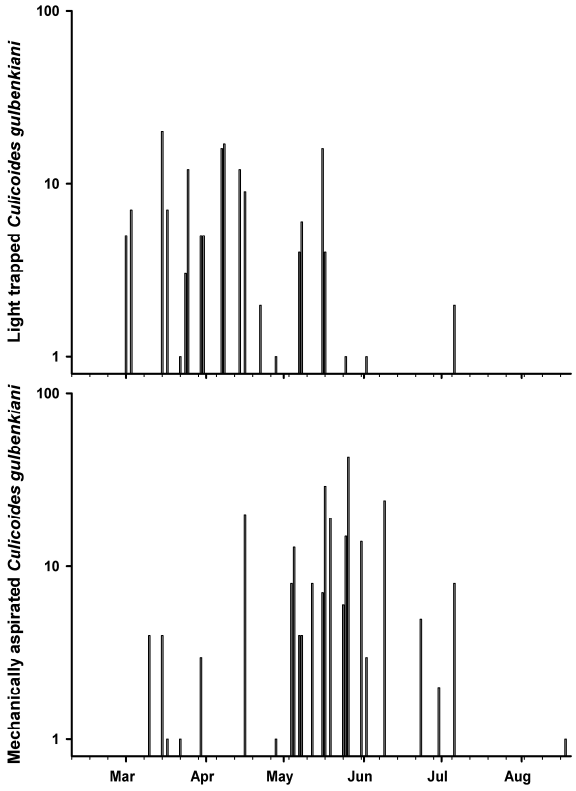

Figure 11: Number of *Culicoides gulbenkiani* collected in light trap in 14 hours and by mechanical aspiration from horses in 20 min between 10 Feb 2010 and 18 Aug 2010.

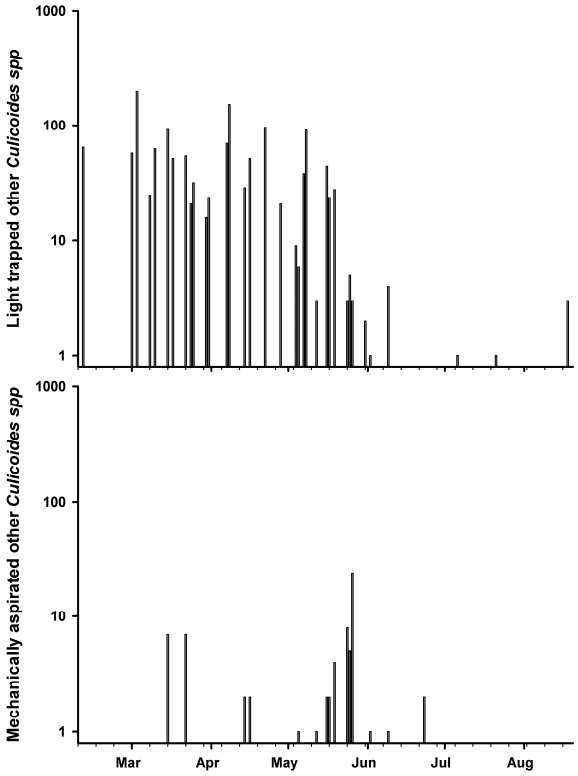

Figure 12: Number of other *Culicoides* spp. collected in light trap in 14 hours and by mechanical aspiration from horses in 20 min between 10 Feb 2010 and 18 Aug 2010.

The light trap that was run concurrent with the mechanical aspiration collected *Culicoides* midges on only six occasions; six *C. imicola* and one *Culicoides* (*Beltranmyia) nivosus* de Meillon. There was marked variation in the apparent appeal of individual bait horses to the *Culicoides* midges with one horse consistently attracting approximately twice as many midges as the others (mean of 916 vs. 400).

Of the 95 *Culicoides* biting midge pools that were tested, AHSV was detected in 11 of 67 light trap pools and in one of 28 mechanical aspiration pools (Table 2). Thus, there was an infection prevalence of 1.35% for midges collected with the light trap and 0.43% for midges collected by mechanical aspiration. The overall field infection prevalence of the *Culicoides*

population was 1.14%. Seven of the AHSV-positive pools consisted of exclusively *C. imicola* only and four of the pools that tested AHSV-positive contained a mixture of *Culicoides* species. One AHSV-positive pool only contained other *Culicoides* species, but no *C. imicola*. It included *C. bolitinos*, *C. nivosus* and *Culicoides* (*Meijerehelea*) *leucostictus* Kieffer among others. The field infection prevalence of *C. imicola* was therefore 0.98%, 1.65% for mixed species and 0.79% for non-*imicola* species. AHSV-positive midges were identified up until 9 June 2010.

Collection method	**Date**	**Number of pools tested**	**Species**	**C_T value**
Light trap	17 Mar	2	292 imicola	33.39
Light trap	22 Mar	1	179 imicola	37.11
Light trap	30 Mar	2	66 imicola	32.84
Light trap	8 Apr	3	200 imicola	33.43
Light trap	19 Apr	2	163 imicola	37.41
Light trap	19 Apr	2	44 non imicola	38.25
Light trap	4 May	1	80 mixed	27.63
Light trap	7 May	2	134 imicola	37.60
Light trap	16 May	2	228 imicola	29.25
Light trap	25 May	1	115 mixed	37.52
Light trap	9 Jun	1	93 mixed	37.28
Mechanical aspiration	16 Apr	1	76 mixed	38.20

Table 2: C_T value of *Culicoides* midge pools tested for AHSV by RT-qPCR

4. Discussion

This study is the first to investigate *Culicoides* midges feeding on horses in sub-Saharan Africa. Previously, biting rates on horses have been investigated in Europe, America and Israel (Mellor and McCraig, 1974; Schmidtmann et al., 1980; Braverman, 1988) predominantly during investigations of the preferred landing sites of different species and the role of *Culicoides* as a cause of EIBH.

Direct comparison of light trapping and mechanical aspiration was complicated by varying optimums in time for collection. Mechanical aspiration was performed at sunset because midges are considered to be most active about that time (Kettle, 1969) and *Culicoides* midges

were observed actively feeding on horses during this period during preliminary runs. Additionally, the reduced visibility after sunset made collections difficult, resulting in the most practical approach of sample collection to be immediately prior to sunset and shortly thereafter. Braverman (1988) experimented with mechanical aspiration at different times of the day and only succeeded in aspirating *Culicoides* midges from a bait horse in Israel from one hour prior to half an hour after sunset. Van der Rijt et al. (2008) also obtained best results at sunset when comparing collections at sunrise, in the early afternoon and at sunset. Light trapping near sunset, during the time period of mechanical aspiration, was ineffective for collection of *Culicoides* midges, as was seen in the total of 7 midges for the entire study period. In a previous study at the study site, midges were predominantly collected in light traps at least 2 h after sunset (Page et al., 2009). This result was concurrent with our findings, which is why only the significantly higher overnight collections were used for comparison.

Culicoides midge catches collected with the two different trapping methods showed marked differences and there was poor agreement between the relative numbers of midges caught on a daily basis (Fig. 9 – 12). The seasonal distribution of *Culicoides* midges also differed; the mechanical aspiration at sunset did not confirm a previously described putative peak vector season during the South African late summer using light traps (Meiswinkel et al., 2004). Numbers of aspirated *C. imicola* were evenly distributed throughout the vector season (Fig. 9). Collections of *C. bolitinos* (Fig. 10) and *C. gulbenkiani* (Fig. 11), however, peaked soon after collections in the light trap diminished.

In general, *Culicoides* tend to have distinct crepuscular activity peaks, and sunset is often a peak host-seeking or flight period (Kettle, 1969; Barnard and Jones, 1980). Still, seasonal temperature differences during fall (May and June) probably led to the relatively high midge collections from the bait horses, and lower collections in the light trap. During cooler weather the body heat of the horses may be more evident to the midges than the UV light of the light trap, favouring mechanical aspiration. More importantly, midge activity probably also decreases nocturnally during cooler weather, and light traps, as shown here, are completely ineffective before sunset. Barnard and Jones (1980) showed that flight activity of *C. sonorensis* is not only influenced by light intensity, but also by other environmental factors with seasonal and diel rates of change leading to variable times of peak activities during the year. Consequently, *Culicoides* midge activity on the horses could have been missed during the hottest summer periods as collections were coordinated by sunset not by temperature. Higher num-

bers of *Culicoides* might have been collected if mechanical aspiration had been conducted after sunset during midsummer. The large and distinct fall peaks in biting activity of *C. bolitinos* and *C. gulbenkiani*, as well as the very significant late-season feeding by *C. imicola* would not have been appreciated at all if light traps were the sole sampling devices.

Most of the *Culicoides* species collected in the present study have been previously identified to occur abundantly near livestock (Nevill et al., 1992b; Meiswinkel et al., 2004). The species abundance, however, differed between the two trapping devices that were compared (Table 1). Although *C. imicola* was the predominant *Culicoides* species on the horses, it was less dominant than expected from the light trap collections. *Culicoides bolitinos* has been proposed previously to be a potential vector for AHSV (Nevill et al., 1992b; Venter et al., 2000; Venter and Paweska, 2007). Its very common occurrence on the horses (28%) in this study supports these observations, and it was relatively more abundant on the horses than one would surmise based on the light trap collections. *Culicoides bolitinos* is susceptible to AHSV infection in the laboratory (Venter et al., 2000) and was the dominant vector during a previous AHS outbreak in the Eastern Free State of South Africa (Meiswinkel and Paweska, 2003). The high percentage of *C. gulbenkiani* detected on the horses (10%), however, is of special interest, and it similarly might have been underestimated as a horse-feeder using only light traps. This species has only been shown to feed on horses by a single positive blood-meal identification study (Nevill et al., 1992b). *Culicoides gulbenkiani* was also previously identified as a potential vector of orbiviruses of livestock in 1992 (Nevill et al., 1992b), and its potential role as vector for AHSV merits further investigation. The third most abundant species collected in the light trap was *C. nivosus* (0.87%), a known bird-feeder (Jupp et al., 1980). Feeding on birds is also common to *C. leucostictus* (0.78%) and *C. pycnostictus* (0.57%), the fourth and fifth most abundant species in the light trap respectively. For many vector groups, host feeding patterns are poorly known or documented, and large numbers of a midge species in a light trap with unknown feeding habits could be misconstrued. The common occurrence of the bird-feeding *Culicoides* in light traps, but absence from horses, illustrates the value of using bait animals to identify epidemiologically important vector suspects. Host feeding patterns of haematophagous Diptera typically reflect both intrinsic host preferences and host availability (relative abundance and overlap in time and space with host-seeking insects). Many *Culicoides* would be expected to be opportunistic feeders. Blood meal analyses of naturally-engorged *Culicoides* collected from the habitat thus would be helpful in

resolving actual feeding prevalence on a specific host such as horses in different ecological settings.

Jones et al. (1977) noted that results obtained from light traps and aspiration of vector insects from bait animals are not comparable due to differences such as skill of the person collecting from animals, ineffectiveness of light traps in some portion of crepuscular vector activity periods, and simply the different attractants- light versus animal-generated cues. Braverman (1988) stated that even though light trap results reflect host-seeking, they also reflect other activities of the insects, such as dispersal or looking for oviposition sites or mates. Gerry et al. (2009) found significant differences between UV-and CO_2-baited traps and mechanical aspiration. These authors concluded that it is vital to conduct animal-based collections along with the baited trap collections in order to interpret the epidemiological significance of the findings. Results of the present study are consistent with these findings, confirming that collection results obtained from a light trap do not reflect biting rates on horses. Mechanical aspiration has the additional advantage of the ability to investigate single animals or only certain areas on that animal, which may be important for other objectives such as EIBH. Disadvantages of aspiration are the influence of weather conditions and the labour intensity of the collection process (van der Rijt et al., 2008). Mechanical aspiration is also more likely to damage individual *Culicoides* midges. Almost 1% of the collection could not be identified because of this, compared to 0% in the light trap. No animal baited trapping had been reported previously in South Africa (Meiswinkel et al., 2004) and with the present study mechanical aspiration has been shown to be a very useful tool in establishing data concerning attack rates of vector insects on animals. Although light traps remain an important monitoring tool for entomologists, they have inherent limitations and mechanical aspiration provides a useful additional or alternative approach to insect collection.

For consistency, one horse was used as bait horse throughout the study period. The randomly allocated second bait horse was enrolled to investigate potential individual differences between horses. The marked difference in midge numbers caught off the trial horses cannot be explained fully. Van der Rijt et al. (2008) reported similar findings using 30 trial horses as bait animals in a drop trap. Variations between individual host animals such as odour of the horse, amount of exhaled CO_2, hair coat characteristics and body heat may influence the attraction of biting midges. The same study conducted baseline experiments regarding the presence of humans. It was shown that humans attracted few or no *Culicoides* compared to horses

and that their presence was therefore negligible (van der Rijt et al., 2008). Still, the time spent close to the horses was reduced to a minimum in the present study and only one investigator collected midges throughout the study period. Previous studies have shown that the presence of animals influences midge catch results in a light trap (Venter et al., 2011). With a correlation factor of less than 0.2, this could not be confirmed in the present study. Another source of potential variability in the present study is the body region sampled, because some *Culicoides* species do attack different body regions (Braverman, 1988), and our study consistently focused on the dorsal aspect favoured by *C. imicola*.

Real-time RT-qPCR has been described to detect AHSV in blood samples by Quan et al. (2010). This protocol was adapted for detection of AHSV in single *Culicoides* midges or midge pools. The present study detected AHSV in 11 out of 96 *Culicoides* pools, resulting in a field infection rate of the *Culicoides* population at Onderstepoort of 1.14% in the year 2010. Since blood-fed midges were excluded from the pools, the high prevalence cannot be attributed to freshly ingested AHSV-positive horse blood. The midges must have acquired and digested the virus previously, and at the time of sampling the virus may already have infected and replicated in the midges. The applied real-time RT-qPCR is quantitative and detected varying amounts of AHSV RNA, evident in the different C_T values. However, it cannot be determined whether this is because AHSV replicated in one positive midge, or because a larger number of midges were AHSV-positive in some of the pools. The calculated field infection rate of 1.14% exceeds previous findings of less than 0.0001% up to 0.005%, which have been reported using virus isolation from midges during AHS outbreaks (Nevill et al., 1992a; Paweska and Venter, 2003; Venter et al., 2006; Venter and Paweska, 2007). The increased sensitivity of RT-qPCR over virus isolation is probably responsible for these observed differences. As part of another project, all horses of the University of Pretoria were bled and tested for AHSV weekly. Throughout the period of the present study, AHSV was only detected in 1 of 52 horses, which was a subclinical case. However, other equids including horses, zebra and donkeys, are kept close to the study location and may have been responsible for infecting the *Culicoides* midges with AHSV. Once appropriate molecular techniques become available it will be interesting to compare the virus serotypes obtained from *Culicoides* midges with those circulating in nearby equine hosts.

Seven of the pools that tested positive for AHSV consisted of *C. imicola* only, the main vector for AHSV in Africa. Four pools consisted of mixed pools, so it is uncertain whether *C.*

imicola or another species was positive for AHSV. Prior to the present study, AHSV had been isolated from *Culicoides* other than *C. imicola* only once. In 1988 (Mellor et al., 1990), AHSV-4 was isolated from three pools consisting of non-engorged female *C. imicola* and from two pools of mixed *Culicoides* species, mainly of *Culicoides pulicaris* (Linnaeus) and *C. obsoletus*; a total of 31 pools were tested. The one AHSV-positive pool in the present study leads to a field infection rate of 0.79% for non-*imicola Culicoides*. This is also significantly higher than previously published findings. It is noteworthy that AHSV was still detected by real-time RT-qPCR in June, when the AHS peak season is considered to be over.

Conclusion

Mechanical aspiration was confirmed to be a most useful procedure to monitor *Culicoides* biting midge occurrence on or around animal hosts. The significant differences detected between light trap midge catches and mechanically aspirated midges provide further evidence that light traps are inaccurate in providing exact prevalence data on occurrence and species identification of *Culicoides* midge vectors on their hosts. The predominant *Culicoides* species attacking horses at the study site were *C. imicola*, *C. bolitinos* and *C. gulbenkiani*. This does not, however, prove the role of these species in the transmission of AHSV. The observed peak of *Culicoides* biting midges on the horses in May, along with the positive RT-qPCR pools that were identified through June, suggests that AHSV transmission may occur even after the "traditional" end of the summer season. These studies emphasize that standardized techniques for measuring the variables of vectorial capacity and vector competence need to be developed and adopted to facilitate interpretation and comparison of data (Mellor et al., 2004).

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Conflict of interest

None of the authors have any material interests that can be construed as a conflict of interest.

Use of real-time quantitative reverse transcription polymerase chain reaction for the detection of African horse sickness virus replication in Culicoides imicola

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Abstract

Despite its important role as vector for African horse sickness virus (AHSV), very little information is available on the dissemination of this virus in *Culicoides* (*Avaritia*) *imicola* Kieffer (Diptera: Ceratopogonidae). This study reports on the applicability of a real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to detect AHSV in dissected midges. A total of 96 midges were fed on AHSV-infected blood, after which one test group was dissected into head/thorax and abdomen segments immediately after feeding and the other only after 10 days of incubation. The majority of the midges (96%) ingested the virus successfully and there was no significant difference between the virus concentration in the heads/thoraxes and the abdomens immediately after feeding. After incubation, virus was detected in 51% of the midges and it was confined to the abdomen in the majority of these. The fact that virus was detected only in the heads/thoraxes of four *Culicoides* midges after incubation suggests the presence of a mesenteronal escape barrier. Replication in the salivary

glands was not shown. An increase of the mean virus concentration in the abdomen after incubation indicates localised viral replication. The real-time RT-qPCR is recommended for further studies investigating the replication and dissemination of AHSV in *Culicoides* midges.

1. Introduction

Small biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) are involved in the epidemiology and transmission of a number of orbiviruses of veterinary importance, including African horse sickness virus (AHSV) with nine known serotypes (Howell, 1962). This virus causes an infectious, non-contagious disease, African horse sickness (AHS), which is endemic in sub-Saharan Africa and can have a mortality rate of up to 95% in susceptible horses.

Based on its confirmed vector status, wide geographical distribution, abundance and host preference for larger mammals, the Afro-Asiatic *Culicoides* (*Avaritia*) *imicola* Kieffer is considered the principle vector of AHSV in South Africa (Nevill et al., 1992b; Meiswinkel et al., 2004). This species is also the most important vector of orbiviruses across vast geographic regions in Africa, the Mediterranean and southern Europe (Mellor et al., 2000). Following ingestion by a susceptible midge, AHSV infects and replicates in cells of the mesenteron before entering the haemocoel and infecting secondary target organs such as the fat body and salivary glands (Mellor, 2000; Wittmann and Baylis, 2000). A number of barriers to arbovirus infection appear to exist in *Culicoides* midges, including the mesenteronal infection and escape barriers and the dissemination barrier. A salivary gland barrier has not been shown to be present in *Culicoides* species (Mellor, 1990; Fu et al., 1999). Studies involving the North American vector *Culicoides* (*Monoculicoides*) *sonorensis* Wirth and Jones and bluetongue virus (BTV) indicate infection of the salivary glands to be an essential prerequisite for the transmission of virus (Jennings and Mellor, 1987). No comparable studies have been performed for *C. imicola* and/or AHSV.

A number of real-time reverse transcription polymerase chain reaction (RT-PCR) assays have been described for AHSV (Rodríguez-Sanchez et al., 2008; Fernández-Pinero et al., 2009; Quan et al., 2010), all with high sensitivity and a detection limit of 0.001–0.15 TCID50 per reaction. A realtime quantitative RT-PCR (RT-qPCR) with a unique approach of using circulating field isolates of AHSV (Quan et al., 2010) has recently been used to determine the infection prevalence of AHSV in *Culicoides* midges. The use of PCR to investigate the replication and distribution of AHSV in *Culicoides* midges has not been described.

The objective of this study was to investigate the replication and dissemination of AHSV in fieldcollected *C. imicola* by feeding, incubating and dissecting individuals and performing real-time RT-qPCR on the abdomens and the heads/thoraxes.

2. Materials and Methods

Culicoides biting midges were collected alive using 220 V Onderstepoort downdraught suction light traps (Venter *et al*. 1998) at various sites near cattle at the ARC-Onderstepoort Veterinary Institute, South Africa (25°39'S, 28°11'E; 1 219 m above sea level). After an acclimatising period of 2 – 3 days at 23.5 ºC and a relative humidity of 50% – 70%, field-collected midges were fed on defibrinated sheep blood containing AHSV serotype 6 at a concentration of 106.1 TCID50/mL through a chicken skin membrane (Venter et al., 1991). After a feeding period of 30 – 40 min the blood-engorged females were separated into two groups: one group was dissected within hours after blood feeding (D_0), whilst the other group was dissected after 10 days' incubation (D_{10}). The blood-engorged females were maintained on a 5% (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 µg streptomycin and 1.25 µg fungizone per 1 mL sucrose solution) at 23.5 °C (Venter and Paweska, 2007). Midges were identified as *C. imicola* by examination of wing pattern. Straight Vanna's microscissors (Agar Scientific, Essex, UK) were used to separate the abdomen (containing the midgut) from the head/thorax (containing the salivary glands). Midges that could not be dissected immediately after feeding or incubation were stored overnight in a refrigerator at 4 °C or in a freezer at –70 °C if stored for a longer period.

The dissected midges were subjected to real-time RT-qPCR following an adaption of the protocol described by Quan et al. (2010). *Culicoides* parts were placed separately in MagNA Lyser green beads (Roche Products, South Africa), containing 300 µL lysis/binding solution (AM8500) from the Ambion total nucleic acid extraction kit (AM1836), to which 2.1 µL β-mercaptoethanol was added, or in 300 µL phosphatebuffered saline. After homogenisation in a MagNA Lyser (Roche Products, South Africa), 100 µL of each sample was mixed with 1 µL carrier RNA, 60 µL isopropanol and 20 µL bead mix, the latter consisting of lysis/binding enhancer and magnetic beads. RNA extraction was performed using either the MagMAX Express Magnetic Particle Processor (Life Technologies, USA) or the Kingfisher Flex Automated Purification System (ThermoFisher Scientific, Finland). Purified water and blood from a clinical case of AHS were used as negative and positive controls, respectively. An aliquot of

5 µL of each extract was mixed with 5 µL primers and probe for part of segment 8, which codes for the structural protein VP7 (Quan *et al*. 2010), to obtain final concentrations of 400 nM for each primer and 180 nM for the probe in the 25 µL reaction. The samples were centrifuged, denatured at 95 °C for 1 min using a PCR machine (GeneAmp 9700, Life Technologies, USA) and rapidly chilled at –20 °C for 5 min. A total volume of 15 µL master mix (12.5 µL 2x RTPCR buffer, 1 µL 25x RT-PCR enzyme and 1.5 µL purified water) was added before the samples were centrifuged again. RT-qPCR was performed using the StepOne Plus Real Time PCR system (Life Technologies, USA) according to the manufacturer's instructions.

Analysis of variance (ANOVA) was used to differentiate between mean cycle threshold (C_T) values. Statistical differences between experimental groups were analysed using Fisher's exact test and/or χ^2 analysis. *P*-values < 0.05 were considered statistically significant.

3. Results

The results of the RT-qPCR assays on the abdomens and heads/thoraxes of 47 D_0 and 49 D_{10} *C. imicola*, respectively, are provided in Table 3. AHSV was detected in 45 (95.7%) D_0 midges, three of which (6%) contained virus only in the head/thorax. There was a significant difference between the number of *Culicoides* that tested PCR positive for AHSV in the abdomen (89.4%) and in the head/thorax (34%). AHSV was detected in 25 D_{10} midges (51%), with a significantly higher number being PCR positive for AHSV in the abdomen (49%) than in the head/thorax (8.2%).

There was a significant ($p < 0.001$) decrease in the number of midges in which AHSV was detected in either the head/thorax or the abdomen immediately after blood feeding (95.7%) than after 10 days' incubation (51%). Based on the C_T values no significant difference was identified in the AHSV concentration between heads/thoraxes and abdomens of D_0 *C. imicola* ($p > 0.05$). Only one of the four positive D_{10} heads/ thoraxes (25%) had a C_T value below the D_0 mean, whereas 18 of the 24 positive abdomens (75%) had C_T values below the mean of D_0.

Test group[a]	**D_0 (*n* = 47)**		
Body segment	Head/thorax	Abdomen	Head/thorax or abdomen
Positive samples	16 (34.0%)	42 (89.4%)	45 (95.7%)
Mean C_T (range)	35.83 (31.76–39.39)	34.67 (31.49–39.27)	–
Number of migdes below mean C_T of D_0	7	23	–

Test group[a]	**D_{10} (*n* = 49)**		
Body segment	Head/thorax	Abdomen	Head/thorax or abdomen
Positive samples	4 (8.2%)	24 (49.0%)	25 (51.0%)
Mean C_T (range)	36.74 (30.94–39.95)	32.52 (26.55–39.60)	–
Number of migdes below mean C_T of D_0	1	18	–

Table 3: Summary of real-time RT-qPCR results for body segments of Culicoides imicola after feeding on AHSV-6 infected blood. AHSV, African horse sickness virus; C_T, cycle threshold for AHSV VP7; [a]One group of midges was dissected on the day of feeding (D_0), whilst the other group was dissected after 10 days' incubation (D_{10}).

4. Discussion

With use of RT-qPCR, AHSV RNA was detected in 95.7% of the *Culicoides* midges assayed immediately after feeding on an AHSV-infected blood meal. In previous studies, where similar infection techniques were used, AHSV was isolated only in 44% – 64% of the midges tested immediately after feeding when using cell culture systems (Venter et al., 2000; Venter and Paweska, 2007). In the present study, AHSV RNA was detected in 51% of the midges assayed after incubation. Previous oral susceptibility studies using identical incubation conditions reported markedly lower virus recovery. Depending on the virus isolate used, results for *C. imicola* ranged from 4.3% to 26.8% (Venter et al., 2000; Paweska and Venter, 2003; Venter and Paweska, 2007). In these studies AHS virions were detected using virus isolation on cell culture systems. RT-qPCR, however, detects viral RNA. This technique has been shown to be substantially more sensitive than virus isolation (Quan et al., 2010), which may explain the higher values reported in the present study.

In most of the D_0 midges in which AHSV was found in the head/thorax, virus was also detected in the abdomen. The three *C. imicola* that tested PCR positive only in the head/thorax

were probably harvested and immobilised whilst still taking up the blood meal. The AHSV loads detected in the heads/thoraxes and abdomens of D_0 *C. imicola* were similar ($p > 0.05$), implying that no virus replication had taken place yet. However, the mean C_T value for the abdomens was lower in D_{10} midges (32.52) than in D_0 midges (34.67). A drop of 3.32 in C_T values implies a 10-fold increase of doublestranded RNA (Quan et al., 2010); the observed decrease of 2.15 therefore reveals approximately five times more viral RNA in the abdomens of D_{10} midges compared to D_0 midges. The results are even more prominent if one looks at the lowest C_T value of the abdomens (31.49 in D_0 and 26.55 in D_{10} midges, respectively). This difference of almost five C_T values indicates more than a 50-fold increase of virus load in the abdomens, which was probably due to virus replication in the midgut cells.

It has been shown that *Culicoides* midges express various barriers that limit virus replication and transmission. The present results clearly illustrate that not all midges in a population are susceptible to infection with AHSV and that some individuals are able to clear the virus to below detectable levels within 10 days after feeding on a virus-infected blood meal. The mesenteronal infection barrier may have played a role in the proportion of D_{10} midges (49%) that were able to eliminate AHSV within 10 days without becoming infected. *Culicoides* midges that were PCR positive in the abdomen but exhibited a C_T value below detectable limits in the head/thorax probably expressed a mesenteronal escape barrier, not allowing the virus to escape from the midgut cells. This result relates to a previous study where 43.6% of *C. sonorensis* exhibited such a barrier to BTV (Jennings and Mellor, 1987). In the present study, only four (8.2%) of the D_{10} midges were PCR positive in the head/thorax, indicating that they expressed neither a mesenteronal escape barrier nor a dissemination barrier. Virus that is present in the head/thorax is presumably located in the salivary glands. All four these midges had a higher C_T value in the head/thorax than in the abdomen (i.e. less viral RNA in the head/thorax), implying that no additional viral replication had taken place in the salivary glands. The salivary glands were not specifically dissected but remained part of the heads/thoraxes. However, this study does not indicate whether this could have influenced the results and secondary viral replication in the salivary glands remains unlikely. The mean C_T value of the heads/thoraxes of the D_{10} midges was not significantly different from that of the D_0 midges ($p = 0.3847$). However, the value was 4.22 units higher than for the abdomens in the former test group, which indicates a substantially lower viral load in their heads/thoraxes. The finding also supports the hypothesis that viral replication did not occur in the salivary glands.

Conclusion

The real-time RT-qPCR used in the present study was an adapted version of the protocol optimised for detection of AHSV in blood and organ samples (Quan et al., 2010). This adapted assay has recently been used to quantify viral loads in *Culicoides* midge pools and now it has been shown to be a very sensitive method for investigating AHSV viral load differences in different body parts of *Culicoides* midges as well. Future studies investigating AHSV replication in *Culicoides* midges should include investigations of AHSV viral load in salivary glands and/or saliva. The authors declare no conflict of interest.

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Authors' contributions

A.J.G. and N.O. were the project leaders, whilst E.G.S., G.J.V. and A.J.G. were responsible for the experimental and project design. The experiments were performed by E.G.S. and C.J. and statistics were performed by G.J.V. E.G.S. wrote the manuscript with contributions from all authors.

Chapter Three: Discussion

Comparison of trapping methods

Culicoides midge catches collected with the two different trapping methods, light trap and mechanical aspiration, showed marked differences. There was very poor agreement between he number of midges caught on a daily basis (Fig. 8 − 11). The seasonal distribution of *Culicoides* midges also differed: mechanically aspirated *C. imicola* were evenly distributed (Fig. 8), however, *C. bolitinos* (Fig. 9) and *C. gulbenkiani* (Fig. 10) showed peak occurrence after collections in the light trap dropped. During May and June, more midges were collected on the horses than in the light trap. This could possibly be explained by change of temperature. During winter with lower air temperatures, the body heat of the horses may be more attractive than the UV light of the light trap, favouring the mechanical aspiration. Midge activity may also decrease during cold winter nights and hence fewer midges are collected overnight. The species abundance also differed between the two trapping devices that were compared (Table 1). Although *C. imicola* was the predominant *Culicoides* species on the horses, it is not as dominantly represented as was to be expected from results in the light trap.

The light trap that was run during the time period that mechanical aspiration was performed caught extremely few *Culicoides* midges, as was to be expected since light traps only attract insects after sunset. The time span around sunset was chosen for mechanical aspiration based on previous reports (Kettle, 1969; Braverman, 1988; van der Rijt et al., 2008) and according to own observations during preliminary collections.

It is known that results obtained by light traps and by aspiration of vector insects from bait animals are not directly comparable, because results are dependent on the efficiency of the investigator who is performing the aspiration, because the light trap is ineffective during morning crepuscular hours and because of the different attractants – light vs. host animal (Jones et al., 1977). Braverman (1988) stated that even though light trap results reflect host seeking, they also reflect other activities of the insects. Gerry et al. (2009) found significant differences between UV light and CO_2-baited traps and mechanical aspiration. These authors concluded it vital to conduct animal-based collections along with the baited trap collections in order to interpret the epidemiological significance of the findings. Results of the present study support those findings and it can be concluded that results obtained from a light trap do not reflect biting rates on horses. Mechanical aspiration has the additional advantage of investi-

gating single animals or only certain areas on that animal, which may be important for other objectives such as finding causal relationships between midge localization and clinical presentation with EIBH. Disadvantages of direct midge aspiration from animals are the influence of weather conditions and the labour intensity (van der Rijt et al., 2008). No animal baited trapping had been reported in South Africa previously (Meiswinkel et al., 2004) and with the present study mechanical aspiration has been shown to be a very useful tool in establishing data concerning attack rates of vector insects on animals. Even though light traps remain an important monitoring tool for entomologists, one must be aware of the shortcomings and consider mechanical aspiration as useful addition or alternative.

Culicoides biting midges

Culicoides imicola was the predominant species collected by both trapping methods. In contrast to light trap collections *C. bolitinos* occurred abundantly on the horses (28%). The latter has already been investigated as possible vector species for AHSV (Nevill et al., 1992b; Venter et al., 2000; Venter and Paweska, 2007). It is susceptible to AHSV infection in vitro (Venter et al., 2000) and was implicated as the dominant vector during an AHS outbreak in the Eastern Free State of South Africa (Meiswinkel and Paweska, 2003). The high percentage of *C. gulbenkiani* detected on the horses (10%) is of special interest. This species has only been shown to feed on horses by one positive blood meal identification previously (Nevill et al., 1992b). It was identified as a potential vector of livestock orbiviruses in 1992 (Nevill et al., 1992b), and its potential role as vector for AHSV needs to be investigated further. The actual and relative numbers of *Culicoides* species collected with the light trap and by mechanical aspiration are given in Table 1.

Previous studies found *C. imicola*, *C. subschultzei*, *C. magnus*, *C. zuluensis*, *C. bolitinos*, *C. pycnostictus*, *C. leucostictus*, *C. nivosus*, *C. schultzei* and *C. enderleini* to be the most abundant species near livestock (Nevill et al., 1992b; Meiswinkel et al., 2004). With the exception of *C. schultzei*, all of these species were also collected in the present study. However, while a total of 14 species comprised 90% of the *Culicoides* midges reported by Meiswinkel et al. (2004), *C. imicola* alone accounted for more than 90% collected by the light trap in the present study. The third most abundant species collected in the light trap was *C. nivosus* (0.87%), a known bird-feeder (Jupp et al., 1980). Feeding on birds is also common to *C. leucostictus* (0.78%) and *C. pycnostictus* (0.57%), the fourth and fifth most abundant species in the light trap, respectively. They may occasionally feed on mammals, as demonstrated when two sero-

types of BTV were isolated from *C. pycnostictus* (Nevill et al., 1992a), but are only opportunistic mammal feeders and were not aspirated from horses in the present study. As it feeds on zebra, rhinoceros (*Ceratotherium simum* or *Diceros bicornis*) and horse dung, *C. sp. #107* was considered to possibly play a role in the epidemiology of AHS (Venter et al., 2000). No individuals of this species were aspirated from horses in this study.

Culicoides biting midges on horses

One horse was consistently used as bait horse throughout the study period and a second horse was randomly allocated for each collection day. The marked difference in midge numbers caught off the individual trial horses cannot be explained fully. Van der Rijt et al. (2008) reported similar findings using 30 trial horses as bait animals in a drop trap. Variations between individual host animals such as odour of the horse, amount of exhaled CO_2, hair coat characteristics and body heat may influence the attraction of biting midges. Mullens and Gerry (1998) collected comparable numbers of *C. sonorensis* from three bait calves, but the engorgement was significantly higher on one of the individuals. This may be due to semiochemicals, which emanate from body, breath and excrement of vertebrae hosts and influence the physiological behaviour of the vectors like mating, breeding and host-seeking. Haematophagous insects have a highly developed olfactory system and semiochemicals may function as attractants, like CO_2 or 1-octen-3-ol, but also as repellents.

In a previous study, baseline experiments were conducted regarding the influence of the presence of humans. It was shown that humans attract few or no *Culicoides* compared to horses and that their presence can therefore be neglected (van der Rijt et al., 2008). Nonetheless, in order to limit any possible interference by the investigator the time spent close to the horses was reduced to a minimum in the present study and only one investigator collected midges throughout the study period. This also limited the potential individual differences regarding vision and ability of identifying *Culicoides* biting midges. Townley et al. (1984) documented that *Culicoides* species in Ireland preferred landing and engorging on the hind quarters, mane, neck, middle and lower limbs of a horse, although attraction to the lower limbs may have been because a draught-type horse with long hair on the limbs was used. Braverman (1988) published similar findings, which led to a focus on the neck and mane, back and hind quarters of the trial horses in the present study. Previous papers have shown that the presence of animals influences midge catch results in a light trap (Venter et al., 2011), but this was not evi-

dent in the present study. The number of non-*Culicoides* insects collected, however, increased with the presence of other animals.

Real-time RT-qPCR

RT-PCR decreases the time needed for running an assay, the manipulation required and the risk of contamination (Rodríguez-Sanchez et al., 2008). One of the advantages of RT-PCR is that it does not involve cultivating a live virus, which would require increased biosecurity. Additionally, results are available in less than 24 hours and since early detection is an important factor for disease control, this is a major advantage compared to virus isolation (Stone-Marschat et al., 1994). RT-PCR also detects early viral infections more reliably (Zientara et al., 1995), which is important because the time between identification of a suspect case and potential confirmation of the veterinary laboratory should be kept as short as possible (Zientara et al., 1993). As RT-PCR was improved over time, it became more and more sensitive, easily surpassing virus isolation. Real-time RT-PCR has significant advantages over conventional RT-PCR, such as increased sensitivity, reduced turn-around time, and reduced carry-over contamination risk (Mackay et al., 2002). Disadvantages remain and include increased cost due to necessary specific equipment and highly trained personnel. The latter might not be available in developing countries, the countries most often affected by infectious animal health diseases (Fernández-Pinero et al., 2009).

A recently published real-time RT-qPCR for detection of AHSV in blood and organ samples (Quan et al., 2010) was adapted to detect AHSV in single *Culicoides* midge parts and in midge pools. Because of its quantitative nature, real-time RT-qPCR detects varying amounts of AHSV RNA, evident in the range of cycle threshold (C_T) values. A low C_T value can be due to a high AHSV titre in one midge or due to a larger number of midges being AHSV-positive. In the present study, the calculated field infection rate for *C. imicola* of 1.14% exceeds previous findings of less than 0.0001% up to 0.005%, which have been reported using virus isolation from midges during AHSV outbreaks (Nevill et al., 1992a; Paweska and Venter, 2003; Venter et al., 2006; Venter and Paweska, 2007). The increased sensitivity of RT-qPCR over virus isolation is probably responsible for these observed differences.

PCR of *Culicoides* midges was until now only used to facilitate epidemiological and surveillance monitoring by interpreting light trap catches. Cêtre-Sossah et al. (2004) first described a PCR assay detecting *C. imicola* among other *Culicoides* species and other insects, and modi-

fied it later into a real-time qPCR assay (Cêtre-Sossah et al., 2008). Determining the prevalence of AHSV in a *Culicoides* biting midge population had not yet been described. The real-time RT-qPCR assay applied in the present study was not only practical for whole *Culicoides* midges and midge pools, but was shown to also be a very sensitive method for individual midge parts. It is recommended that this method be applied in future studies, especially more detailed investigations on AHSV replication in *Culicoides* midges, e.g. dissecting salivary glands only. It is noteworthy that samples that were diluted with phosphate buffered saline (PBS) showed a lower C_T value, hence greater sensitivity, than samples run with lysis buffer solution. Insufficient data was obtained for a meaningful comparison, but at this stage it is recommended to use PBS as standard for future studies.

The method used for dissecting the *Culicoides* midges was found to be very practical. In order to avoid potential cross contamination, scissors and tweezers used to handle and dissect the midges should be decontaminated in bleach in between individual midges and the handling of the midge parts. These sanitary measures were not performed in the present study. However, the results do not give any indication that cross contamination occurred.

AHSV in *Culicoides* biting midges

Two separate experiments were conducted to investigate the relationship between AHSV and *Culicoides* midges. Firstly, large numbers of field collected midges were tested for their infection prevalence. Secondly, individual midges, which had been infected with AHSV in vitro were dissected and investigated further.

Out of 95 *Culicoides* midge pools consisting of up to 200 midges each, 11 pools were positive for AHSV (Table 2). This results in a field infection rate of 1.14% for the *Culicoides* population sampled at Onderstepoort in the year 2010. Blood-fed midges were excluded from the pools, so positive results were not due to freshly ingested AHSV positive horse blood. Positive midges must have acquired the virus previously, and virus may already have replicated in the midges making them infectious to susceptible horses. Seven of the pools that tested positive for AHSV consisted of *C. imicola* only, the main vector for AHSV in Africa. Four pools consisted of mixed pools, so it is unknown whether *C. imicola* or another species was positive for AHSV in these pools. Prior to the present study, AHSV had been isolated from *Culicoides* other than *C. imicola* only once. In 1988 AHSV-4 was isolated from three pools consisting of non-engorged female *C. imicola* and from two pools of mixed *Culicoides* species, mainly of

C. pulicaris and *C. obsoletus*, when a total of 31 pools were tested (Mellor et al., 1990). The one AHSV positive pool in the present study leads to a field infection rate of 0.79% for non-*imicola Culicoides*. This is also significantly higher than previously published findings. It is noteworthy that AHSV was still detected in midges by real-time RT-qPCR in June, when the AHS peak season is considered to be over. This indicates that the virus might be circulating longer than anticipated by veterinarians, possibly even being able to overwinter.

Individual *Culicoides* midges were fed on blood with an AHSV concentration of $10^{6.1}$ $TCID_{50}$/ml. The concentration was purposely set very high to have as many *Culicoides* as possible successfully infected by feeding on the blood. A viraemic animal, however, will have a much lower viral blood concentration. The extremely high percentage of 96% of *C. imicola* taking up AHSV with their blood meal in the present study can therefore only be achieved in vitro and is probably not representative of the infection rate in the field. However, it exceeds previous findings for *C. imicola* of 44%, 47% and 64% for different AHSV strains and 50.8% for AHSV-7 (Venter et al., 2000; Venter and Paweska, 2007). This is again probably due to the higher sensitivity of the real-time RT-qPCR used for detection of AHSV in the present study compared to indirect sandwich ELISA and virus isolation used in the above-mentioned studies.

Virus particles are transported into the abdomen of the midge together with the blood meal, which is why the majority of the midges were AHSV positive in the dissected abdomens on the day of feeding (D_0) (89.4% out of 96%). There was a significant difference ($P<0.0001$) between *Culicoides* individuals being AHSV-positive in the head/thorax (34%) and in the abdomen (89.4%). The vast majority of the midges that were PCR-positive in the head/thorax were also PCR-positive in the abdomen at the same time. In three *C. imicola* individuals, AHSV was detected in the head/thorax only. They might have still been engorging their blood meal. The concentration of AHSV found in head/thorax and abdomen of *C. imicola* did not differ significantly ($P>0.05$), which implies that no replication had yet taken place. The distribution of virus in separate parts of the D_0 midges can be explained solely by ingestion. After 10 days of incubation (D_{10}) only 25 out of 49 (51%) *C. imicola* were positive for AHSV. The remaining 49% were able to eliminate the virus. Previous studies have shown an even lower virus recovery rate: After feeding on AHSV-7 field strains and vaccine strain, only 4.3% of *C. imicola* still carried virus after 10 days of extrinsic incubation (Venter and Paweska, 2007). Paweska and Venter (2003) showed a virus recovery rate of 5.9% for *C. imicola* 10 days after

they were fed with AHSV-6. Higher numbers were obtained when investigating the susceptibility of *C. imicola* to different serotypes. Results ranged from 5 − 26.8%, with the highest value reached with AHSV-8 (Venter et al., 2000). Of the 51% D_{10} *Culicoides* still positive for AHSV in the present study, a significantly higher number was positive in the abdomen (49%) compared to the head/thorax (8.2%) ($P<0.0001$), which is similar to results obtained from D_0 midges. The difference to D_0 midges, however, is seen in the C_T values. The average C_T value of the abdomen was lower in D_{10} midges (32.52) compared to D_0 midges (34.67). A difference of two C_T values equals a 2^2-fold increase of virus concentration. The results are even more prominent if one looks at the lowest C_T value of the abdomens, which varies from 31.49 in D_0 midges to 26.55 in D_{10} migdes, a difference of almost 5 C_T values indicating a 2^5 fold increase of virus. A possible explanation for the increase of virus concentration in the abdomen after 10 days of incubation is virus replication. The incubation period of 10 days has been shown satisfactory in previous studies (Venter et al., 2000; Paweska and Venter, 2003; Venter and Paweska, 2007), so the lack of positive thoraxes cannot be explained by an inadequate period of time for virus replication.

It has been shown before that *Culicoides* midges express various barriers which can limit virus in its transmission. The MIB may have played a role in the midges which were able to eliminate AHSV within 10 days without becoming infected. The increase in C_T values, however, implies that the virus has entered the midgut cells and replicated in them. From the present study it seems more likely that *C. imicola* express a MEB, where the virus is unable to exit the midgut cells, rather than a dissemination barrier, where the virus already entered the haemocoel, but is unable to infect secondary target organs. The present results relate to previous studies where 43.6% of *C. sonorensis* exhibited a MEB to BTV (Jennings and Mellor, 1987). Four *C. imicola* were AHSV-positive in the head/thorax after 10 days, all of which had a higher C_T value in the head/thorax than in their corresponding abdomen (Table 3 − 4). In one single *Culicoides* midge the head/thorax was AHSV-positive with the abdomen not showing any signs of virus. If virus is in the head/thorax it can be presumed that it is located in the salivary glands. This was the case for 4 out of 49 *Culicoides* (8.16%), indicating that 8.16% of the midges did not express a MEB or a dissemination barrier. Since the viral load in the head/thorax is lower than in the abdomen, it implies that there was no additional viral replication in the salivary glands of these midges. It has to be pointed out that the whole thorax was homogenized and examined by the RT-qPCR instead of cleanly dissected salivary glands. Dissection of the salivary glands was not attempted in the present study as it did not

appear to be practical. Even though the salivary glands were mixed with the remaining thorax and thus diluted, it is still considered unlikely that viral replication occurred in the salivary glands. The C_T value of the D_{10} thorax is not significantly different from the C_T value of the D_0 thorax (P=0.3847), giving no indication for viral replication. It also has to be taken into consideration that the abdomen was also diluted, often including the wings, and that unequivocal results were obtained even though the midgut was not specifically dissected.

Chapter Four: Conclusion

This study was the first to perform mechanical aspiration of *Culicoides* midges directly off horses in southern Africa, the endemic area for AHSV. Mechanical aspiration proved to be a very practical and useful technique, catching up to 529 *Culicoides* midges per trial night. Similar to observations reported from other continents, collections obtained with mechanical aspiration differed significantly from collections obtained in a conventional light trap. This, to our knowledge, was also the first study to perform mechanical aspiration on horses with special regards to AHSV. Especially when looking at vector-host interaction, mechanical aspiration should accompany or even replace light trapping. It has been clearly shown in the present study that light traps alone do not give satisfactory results about vector occurrence on the host and should not be relied upon as an only indicator.

In a unique approach, a previously described RT-qPCR assay was adapted for detection of AHSV in *Culicoides* midges. The quick and repeatable assay proved suitable for individual midge parts as well as for large pools of *Culicoides*. By looking at midge pools, the infection prevalence of a *Culicoides* midge population can be determined. Probably due to the high sensitivity of the RT-qPCR, previously published results for infection prevalences of *Culicoides* midges for AHSV have been exceeded.

This study was the first to aim at locating AHSV in its main vector by feeding *C. imicola* with AHSV, dissecting the midges and performing RT-qPCR on the separate midge parts. Most likely due to a mesenteronal escape barrier, only few *Culicoides* midges became infectious for the host. Previous studies have mostly investigated the interactions between BTV and *C. variipennis*. Although the viruses and vectors are closely related, it is important not to draw conclusions from one study and transfer them to another. In order to meet scientific standards, it is essential to conduct unique studies focussing on AHSV and *C. imicola*.

Appendix

Table 4: C_T value of *Culicoides* biting midge thoraxes and abdomens

DAY 0			DAY 10		
Midge No	**Thorax**	**Abdomen**	**Midge No**	**Thorax**	**Abdomen**
1	35.12	32.18	1	-	27.99
2	-	33.11	2	-	39.60
3	36.37	34.13	3	-	-
4	35.06	33.06	4	-	-
5	-	33.35	5	-	39.54
6	-	32.19	6	-	27.77
7	37.40	-	7	-	32.11
8	-	34.03	8	-	-
9	34.82	33.99	9	-	28.72
10	35.98	33.63	10	-	39.58
11	39.39	-	11	-	-
12	-	36.35	12	30.94	29.23
13	-	37.41	13	-	-
14	-	36.64	14	-	32.87
15	-	37.17	15	-	32.70
16	39.24	39.27	16	-	-
17	-	37.47	17	-	-
18	39.39	-	18	-	28.31
19	-	36.99	19	-	-
20	-	37.53	20	-	32.76
21	-	31.49	21	-	31.52
22	31.76	31.76	22	-	-
23	36.06	35.13	23	-	-
24	-	33.80	24	-	-
25	33.44	32.67	25	-	33.36
26	33.45	32.76	26	39.95	-
27	35.88	32.38	27	-	-
28	-	31.78	28	-	-
29	37.57	33.75	29	-	-
30	32.30	33.25	30	-	-
31	-	36.69	31	-	-
32	-	36.10	32	-	38.81
33	-	34.15	33	-	-
34	-	34.31	34	-	-
35	-	-	35	-	33.18
36	-	35.30	36	-	38.70
37	-	33.76	37	-	-
38	-	33.34	38	-	-
39	-	37.79	39	-	-
40	-	35.10	40	-	38.59
41	-	33.61	41	-	-
42	-	36.31	42	-	28.95
43	-	34.56	43	-	31.96
44	-	-	44	38.33	31.58
45	-	38.25	45	-	28.10
46	-	36.03	46	-	-
47	-	35.40	47	-	28.10
			48	37.73	26.55
			49	-	-

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Declaration of independence

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebene Quellen und Hilfen in Anspruch genommen habe.

Pretoria, den 6. Oktober 2011

Elisabeth Greta Scheffer