Detection of African horse sickness virus in *Culicoides imicola* using RT-qPCR

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PREFACE

The research discussed in this dissertation was conducted in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa.

The research conducted and presented in this dissertation represents original work undertaken by the author and has not been previously submitted for degree purposes to any university. Where use was made of the work of other researchers, it is duly acknowledged in the text. The reference style used in this thesis is according to the specifications given by the NWU Harvard Referencing Guide.

Any opinions, findings and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto.

SUMMARY

African horse sickness (AHS) is an infectious, non-contagious arthropod-borne disease of equids. The disease is caused by the African horse sickness virus (AHSV), a member of the genus *Orbivirus*, of the Reoviridae family. It is endemic to sub-Saharan and East Africa and thought the most lethal viral disease of horses. Previous research focused on the diagnosis of host samples rather than detection in the vector. This study focused on detection of AHSV in *Culicoides imicola* pools by the application of real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The aim of this study was the detection of AHSV in field-collected *C. imicola* complex pools in Namibia. The first part of this dissertation focuses on, the performance of the RT-qPCR methodology was determined. Next, the optimal pool for the limit of detection (LOD) of AHSV in *C. imicola* pools was determined by assaying midges in different pool sizes. Midges were fed with AHSV-infected blood and sorted into different pool sizes, with one infected individual per pool. RNA was extracted and prepared for RT-qPCR. The virus was successfully detected and the optimal pool size for the LOD of the virus was determined. A guideline was suggested on the size of pools for accurate and sensitive detection. The second part of the dissertation focused on the application of the RT-qPCR methodology on field collected *Culicoides* in Namibia. *Culicoides* were collected at different sites in Namibia, based on AHS incidence (low, medium and high) over a two-year period (2013–2014), coinciding with the AHS season. *Culicoides* species and abundance at each site were determined. *Culicoides imicola*, the principal vector, was the most abundant species overall. Other implicated AHSV vectors were present at all sites. Collected, sorted and pooled *C. imicola* were assayed with the RT-qPCR methodology based on the pool size determined above. AHSV was detected at all incidence sites and comparisons between sites were made. Windhoek, the medium AHSV incidence site, had the highest number of positives across all sites. There seemed to be a high AHS-incidence time period across sites as well. The importance and application of these results are relevant for AHSV vector identification, vector and virus ecology and for future research on locality-based preventive AHS management.

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LIST OF ABBREVIATIONS

AHS – African horse sickness

AHSV – African horse sickness virus

ANOVA – analysis of variance

BTV – bluetongue virus

cDNA – complementary deoxyribonucleic acid

Cq – threshold cycle

DNA – deoxyribonucleic acid

ENSO – El Niño/Southern Oscillation

LOD – limit of detection

MIQE – Minimum Information for Publication of Quantitative Real-Time PCR Experiments

mRNA – messenger ribonucleic acid

NTC – non-template control

OBP – Onderstepoort Biological Products

OIE – World Organisation for Animal Health (*Office International des Epizooties*)

OVI – Onderstepoort Veterinary Institute

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

RNA – ribonucleic acid

RT-PCR – reverse transcription polymerase chain reaction

RT-qPCR – real-time quantitative reverse transcription polymerase chain reaction

SBV – Schmallenberg virus

$TCID_{50}$/ml – tissue culture infectious dose per millilitre

µl – microlitre

CHAPTER 1: INTRODUCTION

1.1. Background on African horse sickness

African horse sickness (AHS) is an infectious, non-contagious arthropod-borne disease of equids (Boinas *et al.*, 2009; Mellor & Hamblin, 2004; Venter *et al.*, 2000). The disease is caused by the African horse sickness virus (AHSV), an orbivirus, of the Reoviridae family (Mellor *et al.*, 1990; Sailleau *et al.*, 1997; Venter & Paweska, 2007). The virus is spread by haematophagous arthropods, specifically by certain species of biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) (Mellor *et al.*, 2000). *Culicoides imicola* is considered the principal vector. The World Organisation for Animal Health (OIE) considers AHS as a 'notifiable' disease, due to the high mortality rate of susceptible horses and the risk for intercontinental expansion associated with the disease (OIE, 2014).

1.1.1. History of AHS

According to Moulê (1896), AHS was first mentioned in 1327 in the Republic of Yemen. It was suggested that it most likely originated in Africa, after the introduction of horses used in in the exploration of east and central Africa. Later, in 1569, Portuguese explorers in East Africa noted their horses showing suspected symptoms of AHS (Henning, 1956). Donkeys and horses were introduced to southern Africa, in the Cape of Good Hope, in 1652 and reference was made to symptoms of AHS during this time (MacLachlan & Guthrie, 2010). AHS and bluetongue virus (BTV) have played a vital role in the development of veterinary science in South Africa (Van den Bergh, 2009; Verwoerd, 2012). The first outbreak of AHSV occurred in South Africa in 1719 and 1 700 animals were lost to the disease (Henning, 1956). However, the biggest outbreak of AHS in South Africa was recorded from 1854 to 1855, when more than 70 000 horses succumbed to the disease (Henning, 1956).

The first research conducted on AHS in South Africa was by Alexander Edington in 1891. His research covered the cause of the disease, suggesting microbes and fungi (Edington, 1904; 1892). He also isolated an attenuated virus for use in vaccines in 1898 (Edington, 1900). However, Edington's research lost its credibility when he could not substantiate his research findings (Verwoerd, 2012). Some years later, in 1902, veterinarian Arnold Theiler focused his research on the high incidence of AHS on De Onderstepoort farm, later known as the Onderstepoort Research Institute (Verwoerd, 2012). Theiler also developed the first effective vaccine against AHS and BTV (Verwoerd, 2012).

The discovery of the various AHSV serotypes can also be penned to Theiler's name, which was later confirmed by Howell (1962). Serotype discovery led to the development of effective vaccines (Verwoerd, 2012). It is Alexander, however, who did the first AHSV propagation in mouse brains in 1935 (Alexander, 1935) and chicken embryos in 1938 (Alexander, 1938). Theiler and Pitchford suggested an insect vector for the spread of AHSV, but *Culicoides* species were identified as vectors in 1944 by Du Toit (Du Toit, 1944).

Research into the ultrastructure of AHS began in the 1960s. It was discovered that AHS had a distinctive double capsid, the protein coat surrounding the nucleic acids (Els & Verwoerd, 1969). The molecular structure and genetic code of AHS were determined in 1970. These studies showed that AHS is a uniquely structured virus due to the presence of the double-stranded ribonucleic acid (RNA) genome (Verwoerd, 1970). In the 1980s cloning and sequencing of AHS was initiated, which then led to the development of both diagnostic tests and improved vaccines (Verwoerd, 2012).

1.1.2. Epidemiology

AHS is an endemic disease in the tropical and sub-tropical areas of sub-Saharan Africa (Boinas *et al.*, 2009; Guthrie *et al.*, 2013; Mellor, 1993). Regular infection occurs in southern and northern Africa (Fig 1.1) (Boinas *et al.*, 2009). AHS has been reported outside endemic areas and outbreaks have occurred sporadically beyond Africa (Boinas *et al.*, 2009; Guthrie *et al.*, 2013; Mellor, 1993). AHS has been reported in India, Saudi Arabia, Syria, Jordan, Iran, Pakistan, Lebanon, Iraq, Cyprus, Morocco, Spain and Portugal (Boinas *et al.*, 2009; Mellor, 1993; Mellor & Hamblin, 2004). Serotype 9 is widely distributed and mostly responsible for AHS outbreaks beyond African borders (Mellor & Hamblin, 2004). The other AHSV serotypes (1–8) are more restricted to sub-Saharan Africa (Mellor & Hamblin, 2004). Outbreaks of serotype 4 have occurred in Spain and Portugal (Mellor & Hamblin, 2004). African countries have reported the presence of AHSV; specifically Senegal and Kenya suffered AHSV serotype 2 and serotype 7 outbreaks in 2007 (Wilson *et al.*, 2009). Outbreaks most likely occurred due to the movements of nomadic tribes and their animals, and the exportation of equids, specifically zebras from endemic areas (Boinas *et al.*, 2009; Mellor, 1993). Wind is believed to be implicated in the dispersal of *Culicoides* vectors through air currents and, therefore, the spread of the disease (Sellers *et al.*, 1977). Several outbreaks have occurred through this mode of transmission, specifically in the Cape Verde Islands, Cyprus and Spain (Sellers *et al.*, 1977).

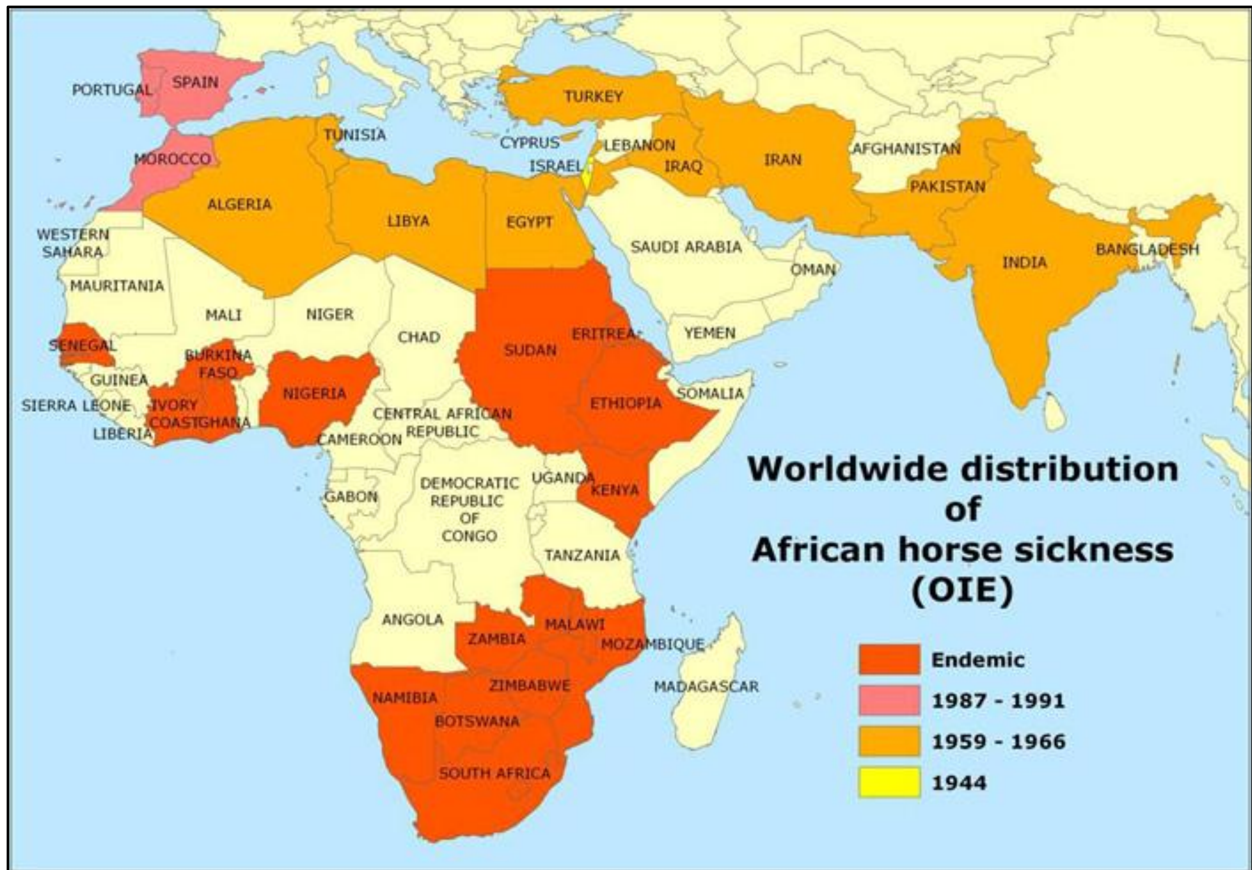

Figure 1.1: Worldwide distribution map of AHS indicating South Africa and Namibia as endemic. AHS endemic areas are indicated in red. Outbreaks in Spain, Portugal and Morocco for the period 1987–1991 are shown in pink. Outbreaks for the period 1959–1966 in northern Africa, the Iberian Peninsula and Middle East are indicated in orange. An outbreak event in 1994 in Israel is shown in yellow (Anon, 2015).

AHSV affects equids, including horses, zebras, donkeys and mules. Zebras are believed to be the reservoir host of the virus and play an important role in the persistence of the virus (Centre for Food Security and Public Health, 2006; Mellor & Hamblin, 2004; OIE, 2009). Horses and mules have a high mortality rate when infected with AHSV (Mellor & Hamblin, 2004). Horses infected with AHSV have a mortality rate of between 70% and 95%. Mules have a mortality rate of between 50% and 70% (Coetzer & Guthrie, 2004), while zebras and donkeys are more resistant to AHSV infection (Coetzer & Guthrie, 2004). Zebras have a mortality rate of about 10% (Coetzer & Erasmus, 1994). Even when infected with AHSV, horses have low levels of viraemia and zebras and donkeys have even lower levels (Hamblin *et al*., 1998). Antibodies against AHSV have been found in African elephants, camels, bovids and various carnivores including lions, hyenas, wild dogs, jackals and cheetahs (Alexander *et al*., 1995; Lubroth, 1992; Mellor & Hamblin, 2004). Outbreaks of AHSV in domestic dogs have also been recorded and was associated with the ingestion of

infected horsemeat (Henning, 1956). However, vector-borne transmission of AHSV to dogs may be possible since AHSV cases have been observed in dogs without close contact with other hosts or the ingestion of infected horsemeat (Van Sittert *et al.*, 2013). Even though the role of these animals (susceptible and non-susceptible hosts) in the transmission and maintenance of the virus is uncertain, it cannot be disregarded (Alexander, 1995; Lo Iacono *et al.*, 2014).

AHS has both a cyclic and seasonal incidence (Scacchia *et al.*, 2009). Epidemics occur in cyclic intervals of drought followed by heavy rain. Baylis *et al.* (1999) have found a strong association between AHS outbreaks and the warm phase of El Niňo/Southern Oscillation (ENSO) in South Africa. ENSO events also correlate with various mosquito-borne epidemics (Baylis *et al.*, 1999). The correlation between AHSV outbreaks and ENSOs may be founded on the increased reproduction of vectors in the presence of heavy rain (Baylis *et al.*, 1999). According to Nevill (1971), the abundance of *C. imicola* is directly related to the amount of rain in the preceding month, with a 200-fold increase in their numbers during above-average rainfall seasons. Furthermore, during drought the vector's normal breeding sites are altered, as water sources are scarce and zebras (the suspected reservoir host) gather near the enduring water sources, bringing vector and host into close contact (Baylis *et al.*, 1999).

The highest seasonal incidence occurs in late summer and early autumn, between February and June (Coetzer & Gurthrie, 2004; Monaco *et al.*, 2011; Gordon *et al.*, 2013). AHSV infection is temperature dependent, where increased temperature leads to increased virogenesis and transmission of AHSV (Mullens *et al.*, 1995; Wellby *et al.*, 1996). Viral replication in midges occurs at a minimum temperature of 15°C. *Culicoides* activity is also affected by temperature (Blackwell, 1997), wind speed and relative humidity (Sinclair, 2007). High temperatures increase vector replication (Baylis *et al.*, 1999; Sinclair, 2007; Gordon *et al.*, 2013).

1.1.3. Pathogenesis

AHS is a disease of the blood and lymphatic vessels and clinical signs are associated with damage to endothelial cells (Coetzer & Erasmus, 1994). Initial multiplication and the onset of primary viraemia of AHSV, upon entry into the vertebrate host, occurs in the lymph nodes. From the lymph nodes, the virus spreads to other organs and cells, where secondary viraemia develops (Mellor & Hamblin, 2004).

There are four types of AHS: the pulmonary ("dunkop") or peracute form, the cardiac ("dikkop") or subacute form, the mixed or acute form and horse sickness fever (Henning,

1956). The severity of the infection depends upon host susceptibility, species, previous immunity and the serotype of the virus. The pulmonary form ("dunkkop") is the most severe with a 95% mortality rate in infected horses. Death may ensue before the onset of clinical signs and it is characterised by respiratory distress, dilation of the nostrils and coughing spasms (Coetzer & Guthrie, 2004). The cardiac form ("dikkop") is characterised by subcutaneous swellings of the head, neck, eyelids, cheeks and tongue; the mortality rate is approximately 50% in infected horses (Coetzer & Guthrie, 2004). The most common form, the mixed form, displays a combination of symptoms of pulmonary and cardiac forms, and death occurs three to six days after infection (Coetzer & Erasmus, 1994). Horse sickness fever is very mild, recovery is common and symptoms are minor. General clinical signs of infection include discharge from respiratory surfaces, fever, excessive salivation and depression (Boinas *et al*., 2009; Centre for Food Security and Public Health, 2006; Mellor & Hamblin, 2004; OIE, 2009).

1.1.4. Aetiology

AHS is caused by the AHSV and the aetiological agent belongs to the Reoviridae family and genus *Orbivirus* (Boinas *et al*., 2009; Venter & Paweska, 2007; Verwoerd *et al*., 1972). AHSV has similar morphological characteristics to other members of the *Orbivirus* genus. Other members include equine encephalosis virus, BTV and epizootic hemorrhagic disease virus (Verwoerd, 1979). AHSV is heat stable and the optimal pH for survival is between 7 and 8.5 (Coetzer & Erasmus, 1994; Mellor & Hamblin, 2004). Nine antigenically different serotypes are recognised (AHSV-1 to AHSV-9) (Howell, 1962; McIntosh, 1958). It is a double-stranded RNA virus and its genome consists of 10 linear segments (Ayelet *et al*., 2013; Grubman & Lewis, 1992; Verwoerd, 1979). The segments encode for polypeptides, including seven structural proteins (VP1 to 7) and four non-structural proteins (NS1, NS2, NS3 and NS3A) (Ayelet *et al*., 2013; Boinas *et al*., 2009; Verwoerd, 1970) (Fig 1.2). The non-structural proteins are responsible for morphogenesis and replication (Manole *et al*., 2012). Two major proteins, VP2 and VP5, compose the outer capsid of the virus particle. This layer is involved in cell attachment and cell entry, as well as antigenic diversity (Manole *et al*., 2012; Mizukoshi *et al*., 1992). The inner capsid comprises two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4 and VP6. VP7 is highly immunogenic (Van Rensburg, 2005) and may play a role during infection of cells by interaction with the cell membrane (Basak *et al*., 1996). The structured proteins (VP1, VP4 and VP6) surround the genome in aligned layers (Manole *et al*., 2012). The AHS virion is approximately 70 mm in diameter and unenveloped (Coetzer & Erasmus, 1994; Polson, 1941).

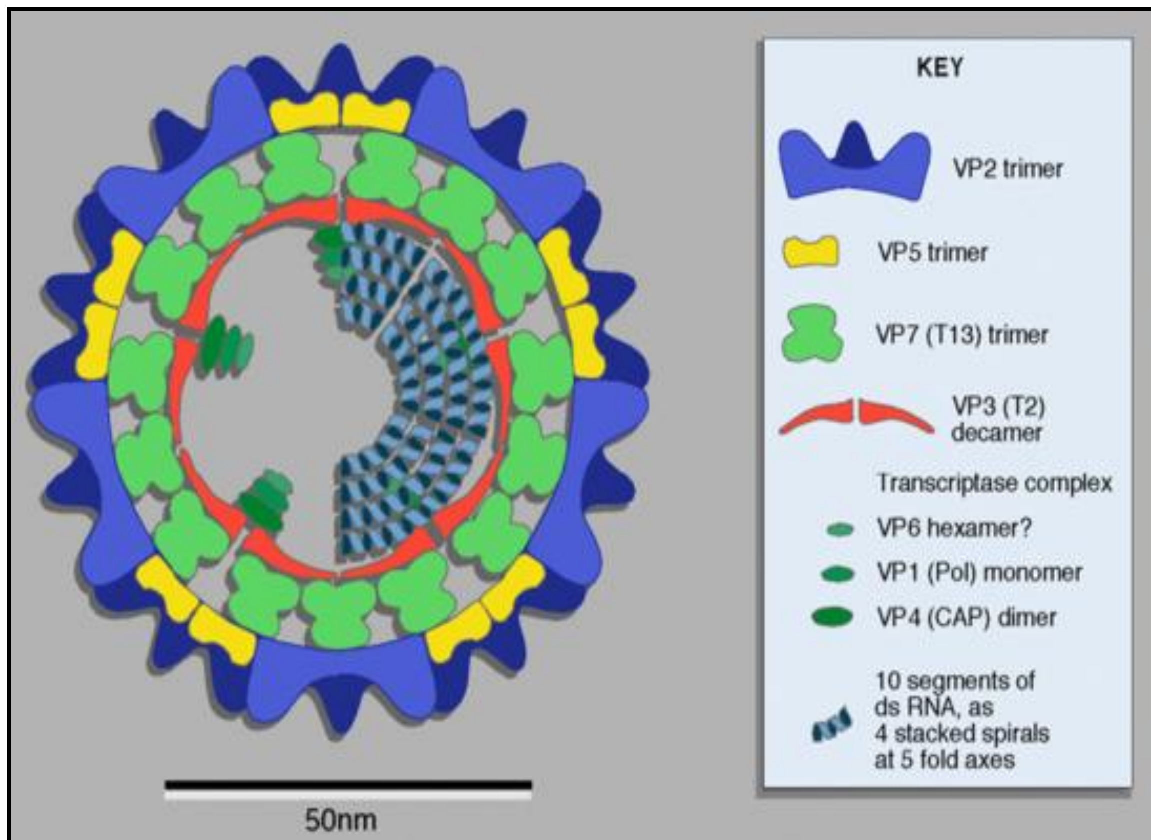

Figure 1.2: The African horse sickness virus structure, indicating the seven structural proteins (VP1 to 7). VP2 and VP5 compose the outer capsid of the virus particle, the inner capsid comprises two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4 and VP6 (Wilson *et al.*, 2009).

1.1.5. Economic importance of horses in southern Africa

Outbreaks of animal diseases pose great risks to all livestock sectors worldwide (Rich & Perry, 2011). The risks include financial impacts of real outbreak situations and precautionary control measures before an outbreak occurs (Rich & Perry, 2011). Disease control can hold many benefits specifically for developing countries (such as South Africa and Namibia), where increased demand for high quality animal products and services can be met through the elimination of animal diseases (Delgado *et al.*, 1999). Control and research in developing countries is essential based on the possibility that these countries may be reservoirs for animal diseases (Winter-Nelson & Rich, 2008).Livestock such as the equine family contribute to the economy in various manners, including food security, draught power, as assets and socially as pets. Disease impacts these contributions, especially in lower-income communities, where contributions may be far-reaching (Perry & Rich, 2007; Randolph *et al.*, 2007). Contrary to popular belief, equines contribute significantly to

livelihoods of communities in Africa (Church, 2014). One example is the use of horse carts in urban and rural transport of both humans and goods (Church, 2014).

The economic importance of AHS and the economic impact on agriculture in South Africa is tremendous. Economic impacts include employment and economic development. South Africa faces a unique challenge for managing profitable animal production in the presence of endemic diseases such as AHS. AHS especially affects the thoroughbred horse industry in South Africa (OBP, 2012). According to Standish *et al.* (2011), the horseracing industry has made a substantial contribution to the national economy of South Africa and the contribution to the gross domestic product in 2009 was R 2.7 billion. The 2011 AHS outbreak in Western Cape, South Africa affected approximately 70 animals and the total laboratory costs amounted to approximately R 850 000. South Africa exports around 200 horses per year and the revenue loss due to this outbreak was projected at R 20 million per year, with foreign investment losses projected at R 200 million per year (Grewar *et al*., 2013).

AHS is a major animal health concern in Namibia (Scacchia *et al*., 2015). The economic effect of AHS in Namibia affects mainly the pedigree horse industry and exportation (Scacchia *et al*., 2009; Scacchia *et al*., 2015). There are about 61 902 horses in Namibia specifically bred for races, sport and the thoroughbred industry (Directorate of Veterinary Services, 2000). Horses are exported to the Arabian Peninsula, Europe and South Africa (Caporale *et al*., 2009). The expected income from exports is estimated at approximately N$ 60 000 per horse (Kazondovi, 2011). In 2011, approximately 1 000 horses were lost to AHS, exports and equestrian events being halted by such mortalities (Kazondovi, 2011).

It seems that AHS outbreaks not only concern horse owners – the effects are much more far-reaching. Outbreaks affects the economy on two levels, namely on farm level, where income activities and natural resources may be affected, and on national level, where disease outbreaks affects livelihoods, animal welfare, environmental concern, trade and tourism, as well as risk management (Rich & Perry, 2011).

1.2. Vectors of AHSV and their importance

AHSV is transmitted by biting arthropods, specifically by certain species of biting midges, *Culicoides* (Diptera: Ceratopogonidae) (Mellor *et al*., 2000). *Culicoides* are among the most abundant yet smallest (1–3 mm in size) of haematophagous insects (Meiswinkel *et al*., 2004a). They are distributed over most of the landmasses, but do not occur in extreme polar regions, the Hawaiian Islands or New Zealand (Mellor *et al*., 2000).

The Ceratopogonidae family consists of 125 genera, with *Culicoides* being one of the haematophagous genera. There are about 1 400 species in the *Culicoides* genus, with over 50 viruses having been isolated from *Culicoides* midges, including orbiviruses such as BTV and AHSV (Mellor *et al.*, 2000; Sinclair, 2007). Female midges are opportunistic blood feeders, preying on an array of animals, with blood-source preferences differing between the species of *Culicoides* (Boinas *et al.*, 2009; Meiswinkel *et al.*, 2004a; Mellor *et al.*, 2000). Larger mammals, especially horses, seem to be favoured especially by *C. imicola* females (Meiswinkel *et al.*, 2004a).

Culicoides imicola (Fig 1.3) and *C. bolitinos* have been shown to transmit AHSV in the field. *Culicoides imicola* is, however, considered the principle vector of AHSV in southern Africa (Nevill *et al.*, 1992; Meiswinkel *et al.*, 2004a). This midge shows exophilic behaviour, preferring outdoor areas, whereas *C. bolitinos* is endophilic (Meiswinkel *et al.*, 2004a). It is believed that *Culicoides* is capable of overwintering, thus allowing AHSV to become endemic (Becker *et al.*, 2012; Boinas *et al.*, 2009; Venter *et al.*, 2014).

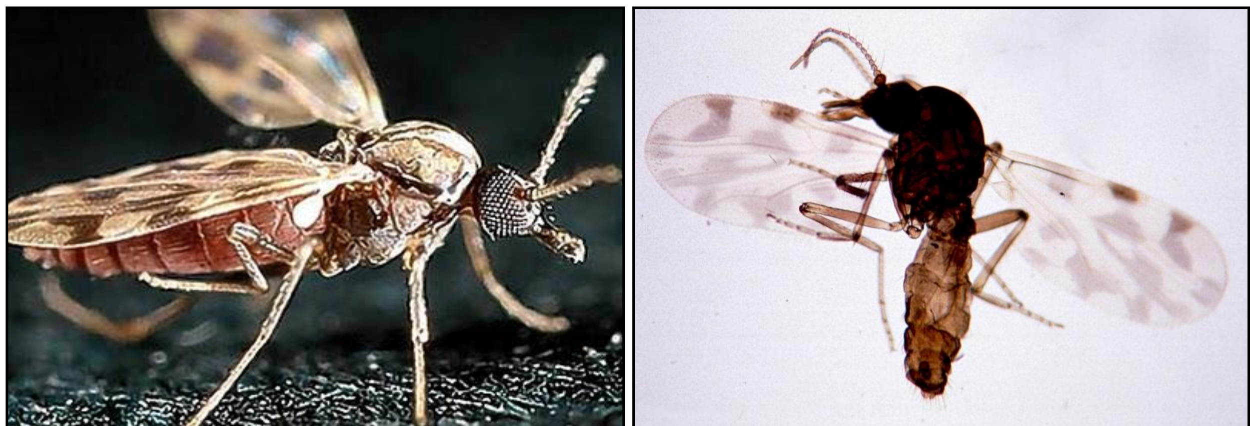

Figure 1.3: Illustrations of female *Culicoides*, both specimens are *Culicoides imicola* (Land Salzburg, 2015; Walker, 2012).

Culicoides can survive cold weather for long periods, suggesting that there is a probability for the survival of AHSV through winter in long-lived midges (Wilson *et al.*, 2009). The year-round presence of *C. imicola* in Spain and Morocco facilitated the survival of AHSV in outbreaks between 1987 and 1991 (Mellor, 1993). However, adult *Culicoides* activity and viral replication can only take place in a suitable climate (Wilson *et al.*, 2009). Overwintering of AHSV occurs when the virus is able to persist in unfavourable climatic conditions (Wilson *et al.*, 2009). Various overwintering mechanisms have been suggested, which include (i) hosts being infected with the virus, but not yet displaying viraemia (Wilson *et al.*, 2009), (ii) persistent infection in zebras and donkeys, which experience longer periods of viraemia compared to horses and have lower mortality rates (Wilson *et al.*, 2009), (iii) *Culicoides* has

been shown to survive in winter months in southern Africa (Becker *et al.*, 2012; Venter *et al.*, 2014), which could prolong the period for AHSV transmission, (iv) vertical transmission in the vector and transplacental transmission in the host, but this is yet to be proved (Wilson *et al.*, 2009), and (v) survival of the virus in an unknown host (Thompson *et al.*, 2012).

The life cycle of *Culicoides* consists of the following phases: eggs, four larval stages, puparia and imago. Cylindrical eggs are laid by the female after a blood meal in a variety of substrates, including degrading vegetation, pools, marshes, animal dung, swamps and soil. (Mellor *et al.*, 2000). *Culicoides imicola* immature stages inhabit moist, organically enriched soil, whereas *C. bolitinos* inhabits the dung of herbivores such as wildebeest, buffalo and cattle (Meiswinkel *et al.*, 2004a). It is estimated that the *C. imicola* female takes about five blood meals during her lifetime (Braverman *et al.*, 1985). Eggs hatch within seven to nine days. The development of the four larval stages is dependent on environmental conditions and is usually between four and twenty-five days. Increased temperatures shorten the life cycle of *Culicoides* and consequently increase the numbers of individuals (Meiswinkel *et al.*, 2004a). Larvae are worm-like and able to swim by means of an eel-like motion. However, Nevill (1967) has found that *C. imicola* pupae are not capable of floating on water surfaces and can thus drown. This explains the need for a semi-moist larval habitat (Braverman *et al.*, 1974). Survival of adult midges varies depending on climatic conditions, usually ranging between 10 and 20 days (Mellor & Hamblin, 2004; Mellor *et al.*, 2000).

Most *Culicoides* have a grey and white wing pattern, which is used to differentiate between species (Meiswinkel *et al.*, 2004a). According to Meiswinkel (1995), the most important species of the *Culicoides* subgenus *Avaritia* is *C. imicola* in the Afrotropical region, due to the transmission of diseases to animals. *Culicoides imicola* has a widespread distribution and seems to be one of the most abundant *Culicoides* species (Meiswinkel, 1997; Meiswinkel *et al.*, 2004a). The *C. imicola* complex includes no less than 12 species (Meiswinkel *et al.*, 2004b). Ten of these have been described (Sebastiani *et al.*, 2001) and eight occur in Africa (Meiswinkel *et al.*, 2004a). The *C. imicola* complex comprises of *C. imicola*, *C. bolitinos*, *C. brevitarsis*, *C. pseudopallidopennis*, *C. nudipalpis*, *C. miombo*, *C. loxodontis*, *C. kwagga* (#107), #30 and #103 (Sebastiani *et al.*, 2001). *Culicoides imicola* has been shown to feed on goats, pigs, cattle, sheep, poultry and horses (Meiswinkel *et al.*, 2004a). The abundance of *C. imicola* associated with livestock poses a risk for susceptible equines (Meiswinkel, 1995). There is a variation in the presence and numbers of *C. imicola* surrounding livestock, as *C. imicola* may be superabundant in some areas and completely absent in others. Presence or absence may be attributed to environmental factors and the presence of hosts (Meiswinkel, 1997, 1998). The presence of AHSV in *C. imicola* is an

important indicator of horses at risk for infection, additonally the presence of AHSV in other vectors could provide information on additional vector species (Scheffer, 2011).

The following *Culicoides* species have also been implicated as vectors for AHSV in oral susceptibility experiments: *C. bedfordi*, *C. brucei*, *C. enderleini*, *C. engubandei*, *C. expectator*, *C. gulbenkiani*, *C. leucostictus*, *C. magnus*, *C. pycnostictus*, *C. sonorensis* and *C. zuluensis* (Bellis, 2013). Other species believed to be implicated in the transmission of the virus are mosquitoes, biting flies and ticks (Coetzer & Guthrie, 2004; Mellor, 1993). Salama (1984) has shown AHSV transmission to dogs and horses by the brown tick, *Rhipicephalus sanguineus*. Although these insects are believed to be possible AHSV vectors, most are only implicated in laboratory studies and their role in the transmission of AHSV remains uncertain (Coetzer & Guthrie, 2004).

1.2.1. Control measures for *Culicoides* and AHSV

Control of *Culicoides* numbers is extremely difficult due to the large number of individuals (Meiswinkel *et al.*, 2004a). An integrated approach is most likely to succeed in controlling *Culicoides* numbers and associated diseases. The most important control measure is the physical protection of the animal against the vector. Approaches that can be used for control include biological control, environmental management (stabling of horses during peak activity) and chemical control (insect repellents) (Carpenter *et al.*, 2008; Venter, 2014).

Medicinal treatment of AHSV-infected horses is limited (Mellor & Hamblin, 2004), thus the need for preventative vaccines and control of the vector. The most effective prevention method is the use of a polyvalent vaccine (Von Teichman *et al.*, 2010). In addition, animal movement regulations is an important aspect of AHS control programmes (Mellor & Hamblin, 2004; Scheffer, 2011). Underreporting of the disease by both private owners and veterinarians contribute to the challenges in controlling AHS (Liebenberg-Weyers, 2015).

In AHS endemic areas, administration of AHS vaccine is the principal preventative measure against AHS infection (Molini *et al.*, 2015). The AHS vaccine used in southern Africa is commercially available and produced by Onderstepoort Biological Products (OBP) (Scacchia *et al.*, 2009; Von Teichman *et al.*, 2010) and is a live attenuated polyvalent vaccine (OBP, 2015; Von Teichman *et al.*, 2010). Not all AHSV serotypes are included in the OBP vaccine – serotypes 5 and 9 are excluded. Serotype 5 can cause severe reaction in vaccinated animals when included in the vaccine (Von Teichman *et al.*, 2010). Serotype 9 is excluded since it is has had a low incidence in South Africa (Von Teichman *et al.*, 2010). The vaccine is administered in two doses, each containing different serotypes (OBP, 2015). The first

dose contains AHSV serotypes 1, 3 and 4, while the second dose contains AHSV serotypes 2, 6, 7 and 8 (OBP, 2015). The doses are administered three weeks apart (OBP, 2015; Scacchia *et al.*, 2009). It is advised that the vaccine be administered in the early summer (OBP, 2015). However, a restriction has been made on the vaccination period by the Department of Agriculture, Forestry and Fisheries in South Africa on 26 March 2015. AHSV vaccination should occur between the period of 1 June and 31 October in AHS protection and infected zones, as outlined in the AHSV Control Policy (Department of Agriculture, Forestry and Fisheries, 2015). It is recommended that additional control measures, such as control of *Culicoides*, be used in conjunction with the vaccine (OBP, 2015).

1.3. Detection of AHSV

In 2012, AHS was added to the OIE's list of notifiable diseases and is listed among Defra's (British Department for Environment, Food and Rural Affairs) major-concern diseases for international monitoring. South Africa and Namibia, as members of the OIE, are required to implement control strategies and surveillance systems for AHS (OIE, 2015; Roberts & Toth, 2013). Tests prescribed by the OIE for AHSV diagnosis for the international movement of animals include complement fixation and enzyme-linked immunosorbent assay. Laboratory detection of AHSV focuses on virus isolation (Sailleau *et al.*, 1997), the detection of antigens (Chuma *et al.*, 1992; Hamblin *et al.*, 1992) or nucleic acids (Zientara *et al.*, 1995; Stone-Marschat *et al.*, 1994).

Due to the nature of the disease, death often occurs in horses before the development of significant antibodies (Rodriguez *et al.*, 1993; Sailleau *et al.*, 1997). This emphasises the need for a rapid diagnostic test, which can reliably detect the presence of AHSV. Therefore, new methods are being developed based on the molecular characteristics of the virus (Sailleau *et al.*, 1997; Zientara *et al.*, 1995).

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is the quantitative process whereby RNA is reverse transcribed to complementary deoxyribonucleic acid (cDNA) (Wiley *et al.*, 2008). Real-time PCR is a rapid assay technique that holds many advantages, including reduced carry-over contamination and increased sensitivity, additionally it can by used for the reliable detection of viral infections (Rodríguez-Sánchez *et al.*, 2008; Zientara *et al.*, 1995). Since RT-qPCR is still being developed and improved, it is expected that sensitivity will increase over time (Scheffer, 2011).

Several studies have covered the use of PCR and real-time PCR for the detection of AHSV from cell cultures and tissue samples. These assays have been optimised specifically for the

detection of AHSV in tissue samples and cell cultures. (Aradaib *et al.*, 2006; Guthrie *et al.*, 2013; Quan *et al.*, 2010; Sailleau *et al.*, 1997). Such a rapid diagnostic technique during a suspected outbreak can prove invaluable (Zientara *et al.*, 1995). Real-time PCR results can be obtained between 24 and 48 hours, whereas virus isolation results may only be available after 15 days (Sailleau *et al.*, 1997).

Virus isolation techniques require highly secure laboratories and the integrity of samples may be affected by transport and storage conditions (Sailleau *et al.*, 1997). This creates a need for a method that does not pose the same problems (Rodriquez *et al.*, 1993). PCR is able to detect AHSV directly from host samples and in samples that have not been properly preserved (Sailleau *et al.*, 1997; Stone-Marchat *et al.*, 1994).

The use of blood samples from hosts for the detection of AHSV is problematic in the application of real-time PCR. Anticoagulants present in the blood may have an inhibitory effect on gene amplification (Sailleau *et al.*, 1997). The use of real-time PCR allows detection of the least virulent strains of the AHSV (Sailleau *et al.*, 1997). Furthermore, the ability of real-time PCR to detect early infection permits the application of control measures as soon as an outbreak occurs (Monaco *et al.*, 2011; Sailleau *et al.*, 1997).

1.3.1. Use of real-time PCR for detection of AHSV

The detection of AHSV in vector samples has been under researched. However, data are available on virus isolations made from collected insects and host samples (Mellor *et al.*, 1990). It is possible to isolate AHSV from *Culicoides* collections, with the inclusion of the principal vector *C. imicola* and other species (*C. pulicaris* and *C. obsoletus*) (Mellor *et al.*, 1990).

Not only are real-time PCR methods available for the detection of AHSV (Aradaib *et al.*, 2006; Guthrie *et al.*, 2013; Quan *et al.*, 2010; Sailleau *et al.*, 1997), but this method can be used to discern between the nine different AHSV serotypes (Weyer *et al.*, 2012). Thus, this methodology has multiple applications for AHSV studies, namely detection (whether in host or vector samples) and serotype identification. Scheffer (2011) detected a gap in the application of real-time PCR for the vector midges of AHSV. Real-time reverse transcription PCR (RT-PCR) can be used for both whole and dissected individuals, as well as for *Culicoides* pools (Scheffer, 2011). The application of real-time PCR on *Culicoides* for the detection of viruses is discussed in more detail in Chapter 2.

1.3.2. Pool screening

The correct evaluation of the prevalence of pathogens in their insect vectors is crucial when assigning control programmes. When prevalence levels are low, as is the case with AHSV, pool screening is more economical than the assaying of vector individuals (Katholi & Unnasch, 2006; Pritchard & Tebbs, 2011). Binomial measurements can be made for pool screening, i.e. positive or negative pools (Katholi & Unnasch, 2006; Pritchard & Tebbs, 2011). The use of insect pools for the detection of viruses in arthropods is a common tool (Hadfield *et al*., 2001; Rasmussen *et al*., 2014; Vanbinst *et al*., 2009) and PCR and RT-PCRs are often used for pool screening (Katholi & Unnasch, 2006). However, the application of pool screening is limited in AHSV detection studies, as pool screening does not provide information regarding the proportion of a pool that may be infected in the case of a positive result (Chiang & Reeves, 1962). Pool screening assays have three approaches: i) individual screening for pathogen presence, ii) screening pools of equal size, and iii) screening pools of varying sizes commonly used for vector-borne pathogens where collection of insects differ over time (Katholi & Unnasch, 2006).

Replications should be done to effectively identify low viral RNA in samples (Hadfield *et al*., 2001). Not doing so could result in inaccurate or false information about the state of the disease in a particular area. Alternatively processing single individuals is not cost or time effective (Katholi & Unnasch, 2006) – thus the need for determining the number of individuals to include in a pool of potentially infected individuals. Determining the size of insect pools does have some bias, but when infection rates are low (as with AHSV) this effect becomes negligible (Katholi & Unnasch, 2006). The super abundance of *C. imicola* and the low infection prevalence emphases the need for a sensitive and accurate method for the detection of AHSV (Venter *et al*., 2006; Walter *et al*., 1980). The sensitivity and specificity of the PCR assay will also be determinant of pool sizes (Katholi & Unnasch, 2006). PCR assays are able to process up to 100 insects in a pool with acceptable sensitivity and specificity (Katholi & Unnasch, 2006). The application of pool screening to vector insects and their associated pathogens are discussed in more detail in Chapter 2.

1.4. Perspective and outline of dissertation

1.4.1. Problem statement

AHS is a non-contagious, arthropod-borne disease primarily affecting equids and is transmitted by *C. imicola* (Meiswinkel *et al.*, 2004a; Mellor *et al.*, 2000). AHS has a high mortality rate in susceptible horses and there is a risk for expansion beyond endemic areas (OIE, 2014). Areas that are theoretically appropriate for AHS transmission is believed to be substantially larger than at present (Wilson *et al.*, 2009). Establishment of AHSV serotypes in western African countries is an indication of the movement of AHS (Diouf *et al.*, 2013; Mellor & Hamblin, 2004). Death often occurs in infected horses before the development of significant antibodies (Rodriguez *et al.*, 1993; Sailleau *et al.*, 1997) and a rapid initial diagnosis during an AHS outbreak is, therefore, vital (Stone-Marschat *et al.*, 1994). The use of vectors to draw information on AHS status may be beneficial, since information can be obtained before the testing of host samples is possible or before the death of animals occurs. This highlights the importance of an AHSV detection tool in *Culicoides* populations.

The first isolation of AHSV from the *Culicoides* biting midges led to the discovery of AHSV vectors (Du Toit, 1944). The detection of AHSV in especially field *Culicoides* is of the essence, since it provides information regarding disease risk in a particular area and can determine possible vectors for AHSV (Scheffer, 2011). However, field-collected vector species of *Orbivirus* may have a relative low infection (Walter *et al.*, 1980).

Several studies have covered the use and optimisation of RT-qPCR for the detection of AHSV in host tissue samples (Aradaib *et al.*, 2006; Guthrie *et al.*, 2013; Quan *et al.*, 2010; Sailleau *et al.*, 1997). The same RT-qPCR assays that are used for the detection of AHSV in horses can be applied to *Culicoides* midges (Scheffer, 2011). RT-qPCR has been applied to AHSV and *C. imicola* for determining viral replication rate and the infection prevalence respectively (Scheffer *et al.*, 2011; Scheffer *et al.*, 2012). Scheffer (2011) detected a gap in the application of real-time PCR for vector midges of AHSV. Real-time PCR has been used for the detection of other viruses, including BTV and Schmallenberg virus (SBV), in *Culicoides* midges (De Regge *et al.*, 2012; Elbers *et al.*, 2013; Vanbinst *et al.*, 2009; Veronesi *et al.*, 2008; Veronesi *et al.*, 2013). RT-qPCR is a rapid analysis method (Guthrie *et al.*, 2013), making it the preferred tool for the detection of AHSV. This application of RT-qPCR in vector midges can be used to determine the presence of AHSV. Such information may be valuable where data are not available on AHSV presence and distribution. However,

no standard operating procedure is available for the processing of *C. imicola* for such an RT-qPCR assay.

According to Katholi *et al.* (1995), PCR assays can be used to detect a pathogen in insect pools, these pools can be used to determine infection prevalence. A pool screening PCR should meet two conditions: firstly, the assay should be able to detect a single infected insect in a pool of uninfected insects, and secondly, a method must be developed to determine infection prevalence (Katholi *et al*., 1995). Due to the lack of such a pool screening technique for the detection of AHSV in *C. imicola,* the focus of this study was on the first requirement.

Furthermore, no standard exists for the number of *C. imicola* individuals per pool to be tested for the detection of AHSV in such a pool screening. Before determining the presence of AHSV in the field, it is important to have a standard to process insects for an assay to obtain accurate results for in-field situations. This limit of detection (LOD) determination in the laboratory is of utmost importance; it would be of no use to process 500 individuals in a pool (with one infected individual theoretically present) and the PCR assay is not sensitive enough for such detection. Additionally, false negatives may be produced (Quan *et al*., 2010) in PCR assays when virus concentrations are at the LOD.

Pool screening is a valuable surveillance tool to determine transmission of virus and the subsequent control programs (Katholi & Unnasch, 2006). It acts as an early-warning tool of occurring transmission (Yamèogo *et al*., 1999). Thus the use of pool screening as a means to determine the presence of AHSV in a particular *C. imicola* population, and consequently a specific area, could be a valuable tool for the implementation of control interventions, as opposed to the normal prescribed diagnostic tests reliant on host samples.

1.4.2. Aim and objectives

The aim of this study was the detection of AHSV in field-collected *Culicoides imicola* complex pools in Namibia using RT-qPCR.

The objectives included the following:

- Determining the sensitivity of RT-qPCR methodology.
- Determining the LOD of AHSV in *C. imicola* pools.
- Determining the presence of AHSV in *C. imicola* using RT-qPCR in Namibia.

- Comparing the presence of the virus in the vector collected at different sites in Namibia.

1.4.4. Outline of dissertation

Chapter 1 is the introduction to the study, which includes the background to the study. It describes the history of AHS, epidemiology, pathogenesis, aetiology and economic importance of AHS. The vectors of AHS and detection methods for AHSV are also discussed. This chapter also includes the perspective and outline of the dissertation.

Chapter 2 includes a description of the use of RT-qPCR for AHSV studies, focusing on *C. imicola*. The results of the sensitivity of the applied RT-qPCR methodology are discussed together with the LOD of AHSV in *C. imicola* complex pools.

Chapter 3 provides a description of AHS research in Namibia. The three sites across Namibia and sampling methods are described. The results for AHSV presence and *Culicoides* abundance are also discussed here.

Chapter 4 provides a conclusion of all the stated objectives.

CHAPTER 2: DETERMINING THE SENSITIVITY OF RT-qPCR AND THE LIMIT OF DETECTION OF AHSV IN *CULICOIDES IMICOLA* COMPLEX

2.1. Introduction

2.1.1. PCR on the *Culicoides* vector

The limited literature with corresponding research objectives and methodology available makes comparison challenging. There are relevant studies where the vector midges themselves were homogenised, RNA extracted and the PCR applied. Mizukoshi *et al.* (1994) and Rodríquez-Sánchez *et al.* (2008) used segment 5 for the amplification of AHSV RNA. This segment encodes for a non-structural protein (NS1). Segment 5 was chosen as the amplification target since it is conserved within all nine serotypes of AHSV (Mizukoshi *et al.*, 1992) and is considered the most efficient target for the detection of all serotypes of AHSV (Mizukoshi *et al.*, 1994). Furthermore, the messenger RNA (mRNA) encoding for the NS1 gene is more abundantly produced than the other segments (Mizukoshi *et al.*, 1994).

Scheffer *et al.* (2011) used RT-qPCR to detect the viral replication rate of AHSV in *Culicoides* midges. Dissected midges were assayed. The adapted protocol used in this study, described by Quan *et al.* (2010) specifically for the detection of AHSV in organ and blood samples, was applied to *Culicoides* midges. Results showed that the entire *Culicoides* laboratory population is not subject to infection with AHSV and that some *Culicoides* are able to clear the virus below detectable levels after feeding, possibly attributed to to an infection barrier in the midge (Scheffer *et al.*, 2011). It was found that real-time PCR detection of AHSV is remarkably more sensitive than virus isolation (Gurthrie *et al.*, 2013; Quan *et al.*, 2010; Scheffer *et al.*, 2012).

In another study by Scheffer *et al.* (2012) the comparison of trapping methods and AHSV prevalence were determined using dissected female individuals. A preliminary study following the adapted protocol of Quan *et al.* (2010) showed that a single infected individual can be detected in a pool size of 200 *Culicoides* midges. The prevalence of AHSV, determined via RT-qPCR, was found to be very low at approximately 1.14%. This field infection rate of specifically *C. imicola* is much higher than previously determined by means of cell-culture-based methods for virus isolation, which indicated that, even during AHSV outbreaks, the infection prevalence in field-collected *C. imicola* was as low as 0.003% (Venter *et al.,* 2006). However, in most outbreak situations in South Africa the superabundance of *C. imicola* will compensate for this relatively low infection prevalence

(Venter *et al.,* 2006). Either way, infection prevalence in midge populations are low and it seems that the detection of AHSV in midge pools might be challenging. Various *Culicoides* species have been assayed using RT-qPCR for the detection of orbiviruses, and specifically AHSV in Namibia, where AHSV was successfully detected in *C. imicola* pools (Goffredo *et al.*, 2015).

SBV is a newly emerged arthropod-borne disease of ruminants in Europe. Many SBV studies focused on the *Culicoides* vector (De Regge *et al.*, 2012; Elbers *et al.*, 2013; Goffredo *et al.*, 2013; Rasmussen *et al.*, 2014; Veronesi *et al.*, 2013). Insect pools were assayed using real-time RT-PCR and pool sizes varied from single midges to 50 individuals per pool. Most studies dissected individuals before assaying. Artificially infected individuals were used for LOD determination and wild-caught individuals for presence/absence data (where there is no certainty of infection) (Elbers *et al.*, 2013). *Culicoides* midges used in these studies differed from those believed to transmit AHSV.

The RT-qPCR detection has also been applied in the detection of BTV, which is closely related to AHSV, in the *Culicoides* vector (Vanbinst *et al.*, 2009; Veronesi *et al.*, 2009). Kato and Mayer (2007) amplified BTV from *C. sonorensis* using RT-PCR. Individuals were artificially infected with BTV and assayed in pools. These techniques were often used in the surveillance of orbiviruses (Nasci *et al.*, 2002). Although the *Culicoides* species and/or virus may differ, the findings of these studies is invaluable due to the lack of such studies in AHSV research.

Determining the infection prevalence in a vector population is an important tool for epidemiological studies and control programmes (Pritchard & Tebbs, 2011). Real-time PCR can also be used to determine other possible *Culicoides* vectors (Vanbinst *et al.*, 2009).

2.1.2. The use of pools

Pool testing has been applied to a variety of insect vectors and their associated pathogens. Magnuson *et al.* (2003) tested mosquito pools (*Aedes triseriatus* for the presence of vesicular stomatitis. Uninfected individuals (10–30 mosquitoes) were added to pools of artificially infected individuals to emulate a natural situation. Hadfield *et al.* (2001) used RT-qPCR to detect West Nile virus in mosquito pools, where the assay was able to detect one artificially infected mosquito in a pool of 50 uninfected mosquitoes. Katholi *et al.* (1995) used pool screening to detect *Onchocerca volvulus* in black flies, where a single infected fly could be detected among 99 uninfected flies. Veronesi *et al.* (2008) used virus positive:virus negative ratios for detection of BTV in *Culicoides* pools, where one infected individual was

added to pools consisting of uninfected individuals. The pools sizes of uninfected *Culicoides* were 1, 5, 10, 25, 50 and 100. Vanbinst *et al.* (2009) tested collected *Culicoides* for the presence of BTV, where an average of 7.5 midges per pool was used (Vanbinst *et al.*, 2009). Scheffer *et al.* (2012) preliminarily showed that AHSV could be detected in a single infected *C. imicola* female in a pool of 200 midges. Goffredo *et al.* (2015) successfully detected AHSV in female *C. imicola* pools using RT-qPCR, with pools consisting of either 50 or 100 individuals. Venter *et al.* (2006) made up pools of 100–500 female *Culicoides* to determine the infection prevalence of AHSV by means of virus isolation. Dilution analysis can also be used to determine the optimal pool size, as done by Mayo *et al.* (2012) to determine BTV infection prevalence in *C. sonorensis*. An increase in pool size will decrease test sensitivity, especially with pools as large as 100 (Williams, 2010). Individuals were dissected before PCR assays and body parts tested separately or selectively (De Regge *et al.*, 2012; Elbers *et al.*, 2013; Rasmussen *et al.*, 2014).

Williams (2010) suggested that if the prevalence of positive samples is more than 30%, pooling is not effective. The size of pools should be large enough to reduce the total number of tests, but should be small enough to allow for accurate positive results (Williams, 2010). Evidence suggests that use of smaller pools sizes with fewer individuals may be more effective in virus detection studies. A smaller pool may be more representative of the actual infection rate in a particular population of *Culicoides* (Vanbinst *et al.*, 2009). Larger pools could increase the risk of false negatives and a dilution effect may be present (Vanbinst *et al.*, 2009). In a natural setting, a collection of insects would contain both infected and uninfected individuals, thus the need to apply the same principle in laboratory tests. Furthermore, the inclusion of uninfected or negative specimens, i.e. male and nulliparous females, within pools can be used as a measure of the specificity of a RT-qPCR protocol (Vanbinst *et al.*, 2009).

2.1.3. Sensitivity and limit of detection

Analytical sensitivity, according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009), is the minimum copy number of target nucleic acid that can be precisely measured with a PCR assay. It refers to the smallest change in concentration that can be detected by the applicable methodology or instrument and it is also known as the slope of the calibration curve (CLSI, 2004; Ambruster & Pry, 2008; PerkinElmer, 2011; Quansys Biosciences, 2013).

Sensitivity is usually expressed as the LOD (Bustin *et al.*, 2009). The LOD is the lowermost concentration of target nucleic acid that can be consistently measured and observed as a positive result. It is the lowest quantity of target nucleic acid that can be discriminated from the absence of that particular nucleic acid target (CLSI, 2004; Ambruster & Pry, 2008; PerkinElmer, 2011; Quansys Biosciences, 2013). When low copy numbers are present, template does not display a normal distribution between replicates. A Poisson distribution is rather assumed, which predicts that 37% of replicates will contain a single copy of template, 18% of replicates will contain two copies and 37% of replicates will contain no copies (Life Technologies, 2011). The Poisson distribution affects both the sensitivity and number of replicates where low copy numbers are present (Rawer *et al.*, 2003). MIQE guidelines (Bustin *et al.*, 2009) define the LOD as the "lowest concentration at which 95% of the positive samples are detected". The LOD can be expressed in the units applicable to the experiment (Bustin *et al.*, 2009). In this study pool size was used as the measure for optimal limit for detection.

Efficiency is also an important element in determining analytical sensitivity and is calculated by use of a standard curve (Qiagen, 2010a). For instance, efficiency would be measured by collecting AHSV, making serial dilutions of known concentrations, performing a RT-qPCR assay and then constructing a standard curve. The slope of this line will then represent the efficiency of the AHSV assay (Bustin *et al.*, 2009). Analytical sensitivity and efficiency are both measures of the performance of the PCR assay (Life Technologies, 2011).

This chapter aims to determine the sensitivity of the RT-qPCR assay for the detection of AHSV and to determine the LOD of AHSV in *C. imicola* pools.

The specific objectives include:

- Determining assay performance.
- Determining the optimal pool size for LOD of AHSV in *C. imicola*.
- Comparing different studies' assay results.

2.2. Materials and Methods

2.2.1. *Culicoides* collection

It is important to consider the adult feeding habits, host preferences, activity and seasonality of *Culicoide*s midges (Boinas *et al.*, 2009). The main tool for monitoring *Culicoides* is the light trap; the type of trap used will be dependent on the objective of a particular study.

Blacklight (UV) traps are a favoured monitoring tool based on the high numbers and diversity of insects it attracts (Meiswinkel *et al.*, 2004a). Light traps can also be used to obtain information regarding virus risk and the transmission of disease (Meiswinkel *et al.*, 2008; Racloz *et al.*, 2008; Rasmussen *et al.*, 2014). *Culicoides* collected with light traps can be used in taxonomic and molecular studies (Meiswinkel *et al.*, 2004a). Infection-associated behaviour of the vector may increase vector capacity (McDermott *et al.*, 2015). It is suspected that BTV infection is present in the eyes of *C. sonorensis*, therefore decreasing visual acuteness and causing aversion to light (McDermott *et al.*, 2015). This is an important consideration when using a light trap for vector collections.

The Onderstepoort Veterinary Institute (OVI) 220 V suction UV-light trap is a benchmark instrument for *Culicoides* collection (Koenraadt *et al.*, 2014). The OVI trap (Figure 2.1) is more sensitive to the collection of a wide variety and large number of *Culicoide*s species (Venter *et al.*, 2009). This tool aids in determining both the presence/absence of AHSV in the *C. imicola* complex. An advantage of using this UV light trap is that insects can be collected live and preserved (Meiswinkel *et al.*, 2004a). However, the use of this trap also has various drawbacks, including that it requires electricity for operation, a scarce resource in rural areas (Meiswinkel *et al.*, 2004a). Additionally, the fan can damage *Culicoides* individuals and there is some bias present with the strong attractant power of the UV light (Meiswinkel *et al.*, 2004a). Sufficient time is required for travelling to outbreak areas and selection of trap locations (Meiswinkel *et al.*, 2004a). Increased travel time may affect degradation of samples before reaching the laboratory. The OVI trap has an attraction range of 2 m and 4 m (Venter *et al.*, 2012).

Culicoides adults were collected alive on 15 and 16 March 2013, at the Agricultural Research Council (ARC) OVI, South Africa (25°39'S, 28°11'E, 1 219 m above sea level) using the Onderstepoort 220 V suction UV-light trap as described by Venter *et al.* (1998). The OVI trap is equipped with a UV light, which attracts insects, particularly *Culicoides* midges. As they are attracted towards the light, they fly through gauze, which prohibits the entry of larger insects, and they are sucked into a collection beaker by the fan (Venter *et al.*, 2009) (Fig 2.1). Traps were operated from sunset to sunrise, when biting midges are most active (Meiswinkel *et al.*, 2004a). Live insects were collected in a 500 mL beaker with no liquid preservatives in the beaker, tissue paper was placed in beaker for the insects to hide from the fan.

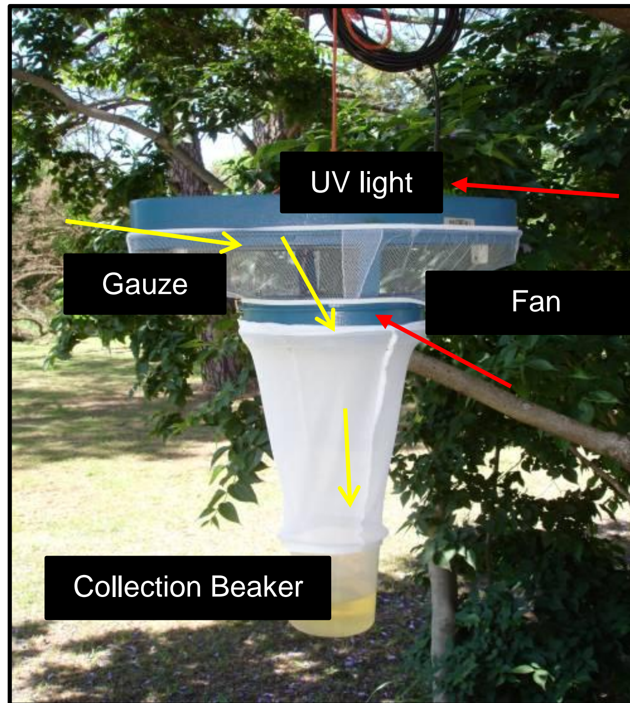

Figure 2.1: The Onderstepoort Veterinary Institute 220 V suction UV-light trap used for insect collections. Yellow arrows indicate movement of insects as attracted by UV light and forced into the collection beaker. Red arrows indicate the position of the UV light and the fan. (AHS equi-link, 2015)

.2.2. Sorting of collections

Culicoides collection and identification is a fundamental requirement in disease monitoring and determining patterns of spread (Liebenberg, 2012). Sorting enhances the efficacy of testing for the virus through the exclusion of uninfected individuals (Scheffer *et al*., 2012). A single light trap catch may contain thousands of *Culicoides* and other insects. Light traps capture a variety of species and sorting, cleaning and identification of collections is laborious and time consuming (Meiswinkel *et al*., 2004a). A subsampling technique was developed by Van Ark and Meiswinkel (1992) to ease the process of sorting. This subsampling technique was applied as outlined by these authors.

Culicoides wing pattern is used for identification (Meiswinkel *et al*., 2004a), an easy task within subgenera (Fig 2.2). However, identifying within species complexes becomes harder, requiring a specialist (Nevill *et al*., 2007). For the purpose of this study, specimens were only identified up to the *C. imicola* complex, hereafter only referred to as *C. imicola,* to ensure increased accuracy. *Culicoides* species were separated from other insects under a stereomicroscope and identified morphologically using the wing picture atlas of Afrotropical *Culicoides* (Meiswinkel, 1996).

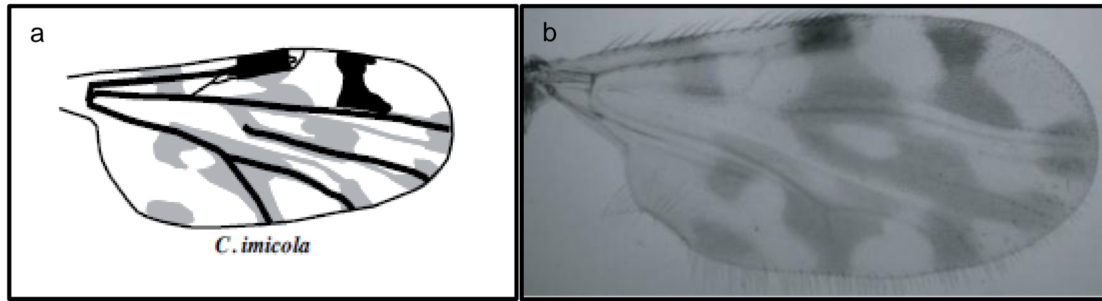

Figure 2.2: The *Culicoides imicola* wing pattern used for identification. a: Diagrammatic wing pattern for *C. imicola* showing distinctive markings (Rawlings, 1996). b: Photograph of *C. imicola* wing (Morag *et al*., 2012).

2.2.3. Artificial infection of *C. imicola* with AHSV

Collected *Culicoides* midges (live specimens) were artificially infected with AHSV. The technique used for blood-feeding was the one-day old chicken-skin membrane technique as described by Venter *et al.* (1991). *Culicoides* were kept at the following conditions: a temperature of 23.5°C, a relative humidity of 50–70% and -1% daylight for a period of 24 hours. During this period no water or nutrients were administered. Midges that survived these conditions were then fed in pools sized between 200–300 individuals for 30–45 minutes. Midges fed on defibrinated sheep blood containing AHSV4 isolates (10^5 $TCID_{50}$/mL) as described by Venter and Paweska (2007). The fresh blood-virus mixture was continually mixed during the feeding process and maintained at a constant temperature of 35.5°C (Venter & Paweska, 2007).

Culicoides samples were separated into different categories, based on parity according to the age-grading technique described by Dyce (1969). The method developed by Dyce (1969) for discerning the status of female *Culicoides* was also used in this study. The method allows the recognition of parous and nulliparous female *Culicoides* based on the presence or absence of red pigment in the abdomen and can be done without dissection. The four categories for age-grading include blood-engorged, gravid, nulliparous (unpigmented) and parous (pigmented) (Fig 2.3). Nulliparous female abdomens do not have a blood meal visible in the gut (Fig 2.3a), thus abdomens lack red pigmentation. Parous females have a moderate pigmentation visible in the abdomen (Fig 2.3b), pigmentation is lighter than that of blood-engorged females. The abdomens of blood-engorged females are coloured bright red (Fig 2.3c). The pigment is concealed after the gut contents begin to darken. Gravid females have visible pigmentation together with the presence of mature follicles (Fig 2.3d). Males do not possess this pigmentation (Dyce, 1969). The age grading of

Culicoides is problematic due to the lack of accurate techniques to do so. It is difficult to make a distinction other than pigmented and unpigmented (Veronesi *et al.*, 2009). According to Braverman and Mumcuoglu (2009) *C. imicola* females that have pigmented abdomens, but were nulliparous, have been found. However, due to the lack of an alternative method, Dyce's method was employed for age-grading.

Figure 2.3: (a-b) a: Unpigmented (or nulliparous) *Culicoides* female. b: Pigmented (or parous) *Culicoides* female (Larska *et al.*, 2013).

Figure 2.3: (c-d) c: Blood-fed *Culicoides* female. d: Gravid *Culicoides* females (Larska *et al.*, 2013).

Fully blood-engorged females were then sorted on a refrigerated chill table and stored individually at -20ºC (Venter & Paweska, 2007). Triplicate groups of unfed nulliparous *C. imicola* were identified and stored in pools consisting of 1, 10, 25, 50, 100 and 200 individuals in 70% ethanol. These pools were used as a dilution, to determine the LOD of a single infected *C. imicola* female in a pool of uninfected females. For viral extraction, one blood-engorged female, fed on the spiked blood, was then added to each of these pools and a nulliparous female removed (Fig 2.4). A negative control consisted of triplicate pools of 200 non-blood-fed nulliparous females.

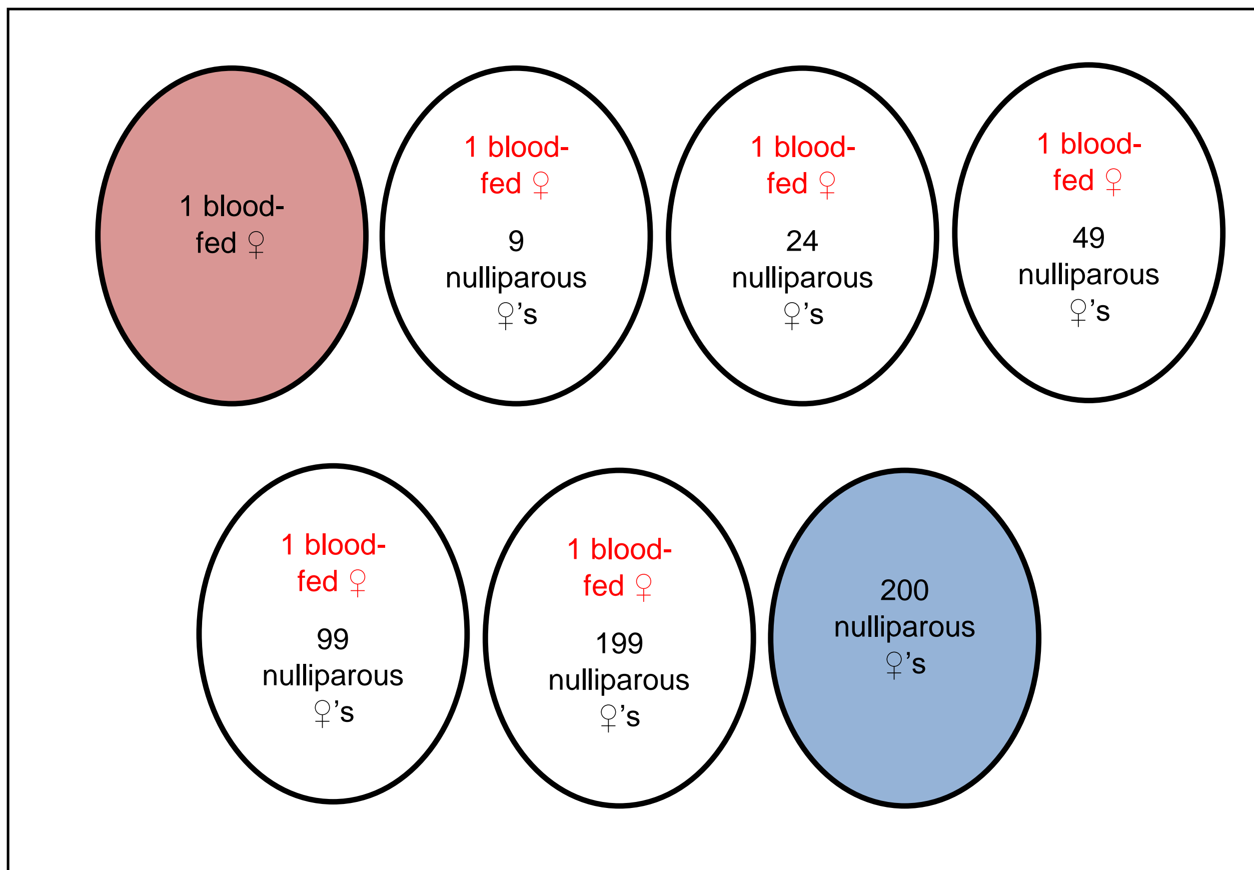

Figure 2.4: The experimental design of pools used for screening. Each pool contained one blood-fed *Culicoides imicola* female (♀), except for the negative control pool. The red pool indicates a single blood-fed *C. imicola* female. The white pools indicate where a single blood-fed *C. imicola* female was added to 9, 24, 49, 99 and 199 nulliparous females respectively. The blue pool had no blood-fed females present.

2.2.4 Viral RNA extraction

Triplicate pools of each pool size were made up before each assay. Before amplification of the AHSV target nucleic acids, the RNA was extracted from the *C. imicola* pools, which consisted of whole individuals, i.e. no dissection was done beforehand. Pools were homogenised in TRIzol® LS reagent (Invitrogen) using 3 mm stainless steel beads for 2 minutes in a TissueLyser (Qiagen). Phase separation was executed with centrifugation. During centrifugation the sample was split into three phase, namely a lower organic phase, a middle phase containing denatured proteins and an upper aqueous phase containing the RNA (ThermoFischer Scientific, 2015). The aqueous phase was removed. The homogenisation and phase separation was done according to the TRIzol® LS reagent manufacturer's instructions. RNA isolation was done with the Qiagen Rneasy® MinElute® Cleanup Kit according to the manufacturer's instructions. The first step in the viral RNA

extraction process was the lysis of tissue (Fig 2.5), where the homogenate was added to the lysis buffer and ethanol. The lysate was then transferred to an RNeasy MinElute spin column (with a silica membrane) (Fig 2.5). The sample was rewashed with ethanol and then centrifuged at 12 000rpm. The membrane was dried with centrifugation. RNA was eluded with RNase-free water (Fig 2.5). The concentration of extracted nucleic acids was determined by use of a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.); dilutions of extracted RNA were made before assaying.

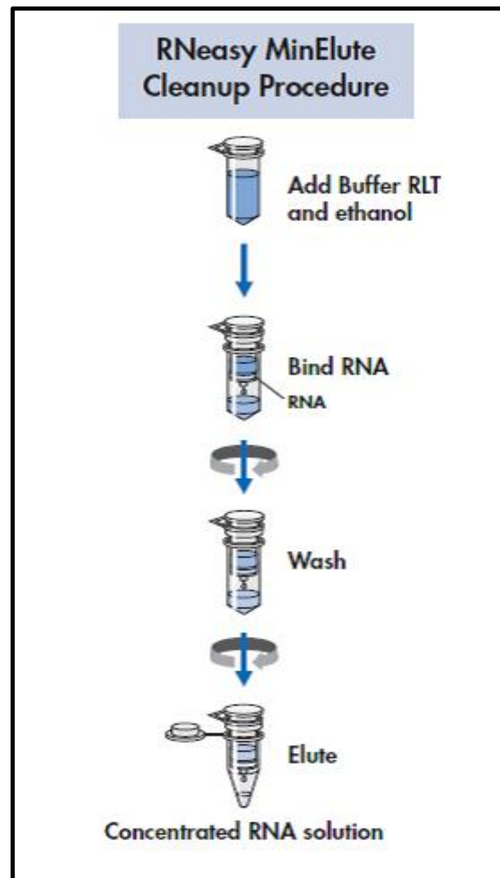

Figure 2.5: Viral RNA extraction process. The lysis, washing and elution steps are shown to obtain high quality viral RNA (Qiagen, 2010b).

2.2.5. AHSV RT-qPCR assay performance

The performance of the RT-qPCR assay was measured with a dilution series. Purified, cultured virus dilutions in phosphate-buffered saline were included in the test rather than different pool sizes of spiked *C. imicola.* The AHSV dilutions were made serially, ranging from 10^9–10 copies, and then assayed. A dilution series aided in determining the LOD (sensitivity) of the assay. A standard curve was constructed using Bio-Rad CFX96 software.

2.2.6. AHSV RT-qPCR assay

A one-step RT-qPCR procedure (according to the MIQE guidelines, Bustin *et al.*, 2009) specific for AHSV was used for the detection of AHSV RNA. Primers and probes from segment 5 were selected (AHSV NS1 forward primer: 5'-CgCAATCTTCggATgTAAgC-3', AHSV NS1 reverse primer: 5'-gCACATACCTTggATCTCTg-3' and 6FAM-TCgCCA+TCC+TCA+TCATCg--BBQ AHSV NS1 Taqman LNA–Tib Molbiol). Primer and probe sequences were complementary to the target sequence which encodes for the NS1 protein. The NS1-gene was chosen as it is considered to be the most efficient target for the detection of all nine serotypes of AHSV (Mizukoshi *et al.*, 1994). Furthermore, mRNA encoding for the NS1 gene is produced more abundantly than other segments (Mizukoshi *et al.*, 1994). The probe used was a TaqMan probe. According to Rodríguez-Sánchez *et al.* (2008) the use of a TaqMan probe in AHSV detection is justified by its high specificity and that it functions as a third primer. The use of a real-time PCR with a TaqMan probe is the preferable test for the detection of AHSV, based on the strength and specificity of this probe (Rodríguez-Sánchez *et al.*, 2008).

The LightCycler® 480 RNA Master Hydrolysis Probes (Roche Diagnostics) and the Bio-Rad CFX96™ Real-Time PCR Detection System were used for the synthesis of cDNA and amplification of target sequences. The advantage of using a one-step RT-PCR kit is the decrease in time needed for the reaction cycle to be completed. Furthermore, the risk of contamination is also reduced. One-step RT-PCRs are also very specific and sensitive (Rodríguez-Sánchez *et al.*, 2008).

Each reaction comprised a total volume of 10 µL, composed of 10 ng/mL of RNA template (extracted RNA subjected to spectrophotometry to determine the concentration of RNA in each sample and diluted to 10 ng/mL RNA) and the LightCycler® 480 RNA Master Hydrolysis Probes (including RT-qPCR buffer – 250 mM bicine/KOH, pH 8.2 (+25°C), 575 mM K-acetate, 40% glycerol (v/v)), activator, PCR grade water and the primers (5 µM of each primer) and probe (6 µM). Each sample was analysed in triplicate. A denaturing step step of 98°C for 30 seconds was applied. The cycling conditions included a reverse transcription step of 61°C for 10 minutes and annealing and extention steps of 95°C for 30 seconds and followed by 44 cycles of 95°C for five seconds and 61°C for 30 seconds. The triplicate positive controls of 10^7 dilutions of the AHSV4 and non-template controls (NTCs) were also included.

The amplification cycle at which the PCR product is first detected, the threshold cycle (Cq value), defines the real-time PCR. Cq values are a quantitative measurement of the initial target nucleic acid (Heid *et al*., 1996).

2.2.7. Data analyses

RT-qPCR results were processed using Bio-Rad CFX 96 software. Some statistical analyses was performed in SAS (SAS Institute Inc., 2015. The SAS System for Windows Release 9.3 TS Level 1MO). Analyses of variance (ANOVA) used in conjunction with Tukey's post hoc analysis were performed to determine statistically significant differences in the number of positive results between the treatments (pool sizes). Confidence levels were also determined to establish the lower LOD. Statistical analysis would identify the optimal pool size for the detection of AHSV in *C. imicola*.

2.3 Results and discussion

The aim of this chapter was to determine the sensitivity of the RT-qPCR assay for the detection of AHSV and to determine the LOD of AHSV in *C. imicola* pools. Firstly, the performance of the assay was determined to see whether it was fitting for the detection of AHSV. The LOD was determined with a RT-qPCR assay applied to *C. imicola* pools. Lastly, a comparison was made with studies with similar methods and results.

2.3.1. AHSV RT-qPCR assay performance

The performance of the RT-qPCR assay was measured by testing the assay with serial dilutions of purified RNA at known concentrations and plotting a standard curve. The standard curve is given in Fig 2.6. The correlation coefficient and efficiency were determined. The standard curve in Fig 2.6 displayed a highly significant linear relationship between Cq values and RNA concentration detected. The known concentrations of the dilution series of AHSV were plotted on the x-axis with the corresponding Cq values on the y-axis. The correlation coefficient (R^2) was 0.997. The correlation coefficient assesses how data fits the standard curve (Life technologies, 2011) and, ideally, an R^2 value of 1 is required.

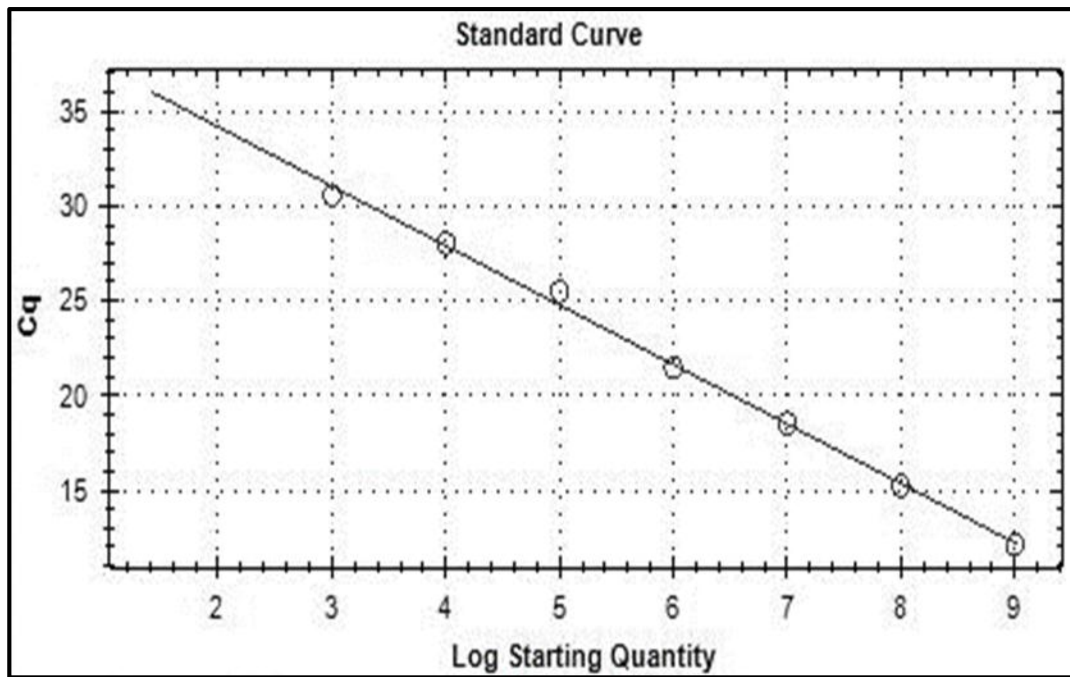

Figure 2.6: Standard curve of dilutions of African horse sickness virus (indicated on the x-axis as 'log starting quantity') versus threshold cycle (Cq) values. The correlation coefficient (R^2) = 0.997 and the reaction efficiency = 108.3%.

The LOD for the target can also be obtained from the standard curve by associating the maximum Cq value (y-intercept) with the minimum log starting quantity (x-intercept). This gives an indication of the possible range of detection of the assay for AHSV RNA. The lowest target level is usually defined between two to ten log copies (Life Technologies, 2011). The y-intercept was 40.467 and Cq values above this value were considered negative results. The LOD for the target was found to be approximately 10^1. Detection was not possible with this low number of target copies. The 10^1 dilution was detected as a negative in the RT-qPCR reaction. Thus the assay is not able to detect AHSV below 10^2 copies of target RNA.

During each cycle of the PCR reaction doubling of the DNA molecules should ideally occur at a maximum of 100 % (Bustin *et al.*, 2009). The efficiency of the reaction was found to be 108.3% and is displayed in the slope of the standard curve, which was -3.138. Reproducible and accurate results are obtained with a slope of between -3.58 and -3.10 or an equivalent efficiency of between of 90% to 110% (Life Technologies, 2011).

Overall the parameter information provided by the standard curve showed that the RT-qPCR reaction was efficient and that there was a correlation between Cq values and target nucleic acids. The high quality of the performance of the assay indicated that it can be successfully and accurately applied for the detection of AHSV and can thus be effectively used for the in detection of AHSV in *C. imicola* pools.

2.3.2 AHSV RT-qPCR results

A total of 258 pools of *C. imicola* were assayed. The results of RT-qPCR on the different pool sizes, varying from 1 to 200 female *C. imicola* individuals per pool, are given in Table 2.1. Pool sizes differed in order to determine the optimal pool size for the detection of a single infected *C. imicola* female amongst uninfected females. Pools were made up of one artificially infected *C. imicola* female and the remaining *C. imicola* midges were non-blood fed as indicated in Fig 2.4. Table 2.1 shows the number of replicates tested for each pool size and the number of pools in which AHSV RNA was detected. In a natural situation, collections of insects may contain both infected and uninfected individuals within the same pool. The same principle was applied to laboratory experiments. This was done to determine the optimal pool size in the presence of a dilution effect, i.e. uninfected individuals, as seen in a natural setting. The inclusion of uninfected females within pools can be used as a measure of the specificity of an RT-qPCR protocol (Vanbinst *et al.*, 2009).

Triplicate pools were used for viral RNA extraction and during the RT-qPCR reaction each of these pools were analysed in triplicate again. A triplicate series originating from a single pool may not all be positive or negative, since the target molecule may not be present in all three replicates, resulting in an uneven distribution of either positive or negative results. This phenomenon is known as a Poisson distribution (see Section 2.1.3). It can thus be assumed that if enough replicates were done, 55% of the replicates would be positive (37% with one copy template and 18% with two copies of template), to effectively minimise the effect of low copy numbers. Replicates are done to effectively identify low viral RNA samples (Hadfield *et al.*, 2001). The Poisson distribution affects both the sensitivity and number of replicates where low copy numbers are present (Rawer *et al.*, 2003).

The mean Cq value for each individual pool size is shown with a confidence interval of 90%. This value was calculated for each pool group using the Cq values for pools in which AHSV RNA was detected.

Table 2.1: Number of *Culicoides imicola* replicates for each pool size. The percentages of positive pools and the mean threshold cycle (Cq) values are also given. Different letters indicate significant differences between pool sizes at $p<0.05$.

Pool size	**1[a]**	**10[a]**	**25[a]**	**50[b]**	**100[a]**	**200[b]**	**Control 200**
Number of pools tested	52	44	44	44	34	38	54
Positive pools	31	22	14	10	6	4	0
Positive pools (%)	59.6	50.0	31.8	22.7	17.7	10.5	0
Mean Cq value of positive pools	30.75	31.12	32.30	33.72	32.92	35.78	-
Confidence interval (90%)*	0.4727	0.3588	0.2037	0.1288	0.0798	0.0368	-

*Minimum interval for detection.

The mean Cq value tended to increase as the pool size increased. A possible dilution effect may be present, where one infected female individual (with the viral RNA) may become swamped by the increasing number of uninfected females and thus a larger concentration of *Culicoides* RNA. Cq values differed between pool sizes, possibly as a consequence of the RT-qPCR detecting fluctuating amounts of AHSV RNA. Cq values above 40 were considered negative results (see y-intercept in Section 2.3.1). AHSV RNA could be detected in all the pool sizes, even in a large pool of 200 individuals. This means that the RT-qPCR reaction was sensitive enough to detect a single infected *C. imicola* female in a pool of 199 uninfected *C. imicola* females. This is in alignment with Scheffer *et al.* (2012), who showed that detection of AHSV in a *C. imicola* female is possible in a pool size of 200.

However, the frequency of AHSV RNA detection declined with increasing pool size. AHSV RNA could be detected in single infected individuals in 59.6% of the samples. A result of 100% detection for single infected individuals was desired. However, obtaining such ideal results is difficult, possibly due to low virus titre in a blood meal, a low volume blood meal taken by an individual or the uneven distribution of target molecules in replicates. It is also important to consider that not all midges in a tested population might have been susceptible to AHSV infection and that some *Culicoides* are able to clear the virus below detectable levels after feeding, possibly attributed to to an infection barrier in the midge (Scheffer *et al.*, 2011). This may have affected the prevalence of positive results.

In the pools of 10 individuals with one blood-engorged midge, AHSV RNA could only be detected in 22 (50.0%) of the 44 pools tested. As the pool size increased and a dilution effect was found, positive results decreased accordingly. In larger pools consisting of 25, 50 and 100 midges, 31.8%, 22.7% and 17.7% tested positive respectively. In the largest pool

size of 200 only four (10.5%) out of 38 pools tested positive for AHSV (Table 2.1). No virus was detected in the negative control pools consisting of 200 nulliparous females. The indication of a Cq value for both the negative and non-template control replicates of 0.00 is a positive indication of the success of both the RNA extraction and isolation and the RT-qPCR protocol. The absence of a Cq value for the NTC is an indication that no contamination took place during the reaction. A lower Cq value (as seen with smaller pool sizes in Table 2.1) indicated a larger starting quantity of viral RNA (Bustin & Mueller, 2005). The Cq value for the positive controls were much lower compared to all pool sizes. The positive control's fluorescent signal was detected much earlier than other samples, which was caused by the higher starting quantity of AHSV RNA in positive controls compared to the other pools' treatment replicates.

The numbers (replicates) per pool size tested differed. These differences were caused by the removal of anomalies. As seen in Table 2.1, fewer replicates were present as the pool size increased. More inconsistencies became apparent with the larger pool groups. Here the sensitivity and specificity of the RT-qPCR come into play. It is possible that the accuracy of the specific method is lower in large pools.

Statistically significant differences between pool sizes were calculated using ANOVA and Tukey's post hoc analysis. The test facilitates the identification of an optimal pool size for AHSV detection. As already mentioned, limited literature is available on the detection of AHSV in *C. imicola* using RT-qPCR. Furthermore, no level of detection or pool size for the effective detection of a single AHSV infected female is available. There is also no standard operating procedure available for the testing of *Culicoides* pools for AHSV presence and this study, therefore, contributes to the development of such a procedure. Thus, comparison of these data with previous studies' results emphasises the importance of these results.

The ANOVA indicated highly significant differences between the pool sizes ($p=0.0006$) (Table 2.1). Pool sizes of 1, 10, 25 and 100 did not differ significantly and the same was true for pool sizes of 50 and 200. However, pool sizes of 1, 10, 25 and 100 differed significantly from pool sizes 50 and 200. The significant differences between pool size 50 and pool sizes 1, 10 and 25 may have been caused by low virus titre in blood meal or a low blood volume taken by individuals in pool size 50. The RT-qPCR detecting fluctuating amounts of AHSV RNA may also have contributed to the differences as may low copy numbers (Poisson distribution). The relatively low number of replicates could have been the reason for this apparent irregularity and further research in this area is needed.

The confidence interval showed that the percentage positives will be between the upper and lower boundaries 90% of the time. This study focused on the lowest confidence interval,

since the LOD was desired. The lower confidence interval showed that the percentage positives in a *C. imicola* population possibly infected with AHSV is not below the values displayed in Table 2.1 for each pool size, 90% of the time. It has been found that the confidence interval narrows as the pool size increases (Katholi *et al.*, 1995). If samples were repeated, the percentage positives for a pool size of 1 would not be below 47% (0.4727) 90% of the time, but the percentage positives for a pool size of 200 would not be below 4% (0.0368) 90% of the time.

The mean Cq value for each pool size was used to derive the approximate log starting quantity of target cDNA from the standard curve in Fig 2.6 The relative amount of starting target cDNA declined as the pool size increased, by the dilution effect. Pool size 1 and 10 had the same approximate starting quantity of AHSV RNA (10^3 copies). Pools of 25, 50 and 100 had the same approximate starting quantity of AHSV RNA with between 10^2 and 10^3 copies. The pool size of 200 had an even lower log starting quantity of AHSV RNA. The pool size of 200 starting quantity (10^2–10^1) corresponded with the sensitivity (LOD) determined in the RT-qPCR performance assay. This may explain the low number of positive results with this pool size. With such low copy numbers, a Poisson distribution of templates between replicates may be present. The means were taken into account and individual log starting quantities may have differed due to the fact that the RT-qPCR detected varying amounts of viral RNA.

The MIQE guidelines (Bustin *et al.*, 2009) define the LOD to be the “lowest concentration at which 95% of the positive samples are detected”. However, as seen in the results above, this was not achieved. There may be various reasons for this. One being the effect of low copy numbers, i.e. the Poisson distribution. Assuming the Poisson distribution of templates in replicates, 55% of replicates are expected to be positive. A pool size of 1 had 59.6% positive and a pool size of 10 had 50.0% positive results. This is an indication that the number of replicates for these two pool sizes were satisfactory for AHSV detection. Theoretically it implies that more replicates are required for the other pool sizes to minimise the effects of low copy numbers. However, in practice this not so simple. Other factors such as assay sensitivity, cost efficiency and the number of individuals available for processing need to be considered.

Deciding on the optimal pool size for the detection of AHSV in a single infected *C. imicola* in a pool of uninfected individuals is complex. Various experimental parameters need to be taken into account. These include statistical analysis, Cq values, Poisson distribution, cost effectiveness, starting quantity of target nucleic acids, assay sensitivity, number of individuals available and time efficiency. Statistical analysis was not able to pinpoint the

optimal pool size for detection, but rather provided a guideline. The other parameters gave similar results – not a particular pool size but a guideline to which pools may be more suitable for optimal detection.

Smaller pool sizes have been shown to be more effective in virus detection studies (Vanbinst *et al*., 2009). Many studies make use of smaller pool sizes for detection purposes (De Regge *et al*., 2012; Hadfield *et al*., 2001; Magnuson *et al*., 2003; Rasmussen *et al*., 2014; Vanbinst *et al*., 2009). Table 2.2 is a representation of the guidelines provided by experimental parameters based on which pools sizes may be suitable for the optimal pool of detection. Blue shading suggests a 'safe' pool size, while red shading pool sizes are not recommended. The LOD can be expressed in the units applicable to the experiment (Bustin *et al*., 2009). Pool size was used as the measure for optimal pool for detection. For the purpose of this study, a pool size of below 50 was considered a good recommendation for AHSV detection. Using a pool size of 50 or below enables the detection of AHSV with satisfactory reliability and efficiency.

Table 2.2: Experimental parameter guidelines for optimal pool for detection. Blue shading suggests a 'safe' pool size, while red shading pool sizes are not recommended. Pool sizes are represented by 1, 10, 25, 50, 100 and 200 respectively. Cq: threshold cycle.

1	10	25	50	100	200	Parameter
[blue]	[blue]	[blue]	[red]	[red]	[red]	Tukey's test (anomaly considered)
[blue]	[blue]	[red]	[red]	[red]	[red]	Poisson distribution
[blue]	[blue]	[blue]	[blue]	[blue]	[blue]	Acceptable mean Cq value
[blue]	[blue]	[blue]	[blue]	[blue]	[red]	Assay sensitivity

These pool screening results could be obtained within a day; rapid results translate into rapid action during outbreaks. A rapid initial diagnosis during AHS outbreak is vital (Stone-Marschat *et al*., 1994). RT-qPCR diagnosis of AHSV in vector samples can provide such a diagnosis. It can be used as a preliminary warning of a potential outbreak situation. Due to the rapid nature of the disease, death often occurs before the development of significant antibodies (Rodriquez *et al*., 1993; Sailleau *et al*., 1997). Thus, using vectors for detection could be beneficial, since information can be obtained from vector assays before testing of host samples is possible.

Nevertheless, there are limitations with RT-qPCR detection in vector samples. Only a certain number of individuals can be included in pools, but pool screening is economical (Katholi & Unnasch, 2006). Unfortunately, with pool screening the proportion of a pool that might be infected in the case of a positive result cannot be determined (Chiang & Reeves, 1962).

However, if studies are focused on presence/absence data, this should not be a problem. The detection of AHSV in *C. imicola* pools of varying sizes has shown that it is a successful pool screening method. This tool can, therefore, be used in field-detection studies.

2.3.3 Comparison of AHSV detection studies in *Culicoides*

Table 2.3 summarises the mean Cq value for different pool sizes for three different studies. This study is included and demarcated by the grey rows. The other studies were by Scheffer *et al.* (2011) and Scheffer *et al.* (2012). The purpose of the study by Scheffer *et al.* (2011) was to determine the viral replication rate of AHSV in *C. imicola* with the use of real-time PCR. Scheffer *et al.* (2012) aimed to compare the most efficient trapping method for the collection of *Culicoides* midges and the determination of the prevalence of AHSV in *Culicoides* populations. Goffredo *et al.* (2015) also tested midges of various species for the presence of orbiviruses, including *C. imicola* for the detection of AHSV, but Cq values are not available for comparison. Pool sizes in this study were made up of 50 and 100 individuals respectively.

The pool sizes ranged from a single infected individual to a pool size of almost 300 *Culicoides* vectors, with one infected individual. It is important to note that the midge samples used in this study were whole midges, whereas the midges in the other two studies were dissected and segments were tested separately. Scheffer *et al.* (2011) showed that there is no significant difference in virus concentration (immediately after feeding) between the head/thorax and abdomen of dissected midges. With the use of whole midges in this study, the omission of dissection contributed to the time efficiency of the process.

The trend in Table 2.1, where the Cq values increased with increasing pool size, was not clearly discernible in Table 2.3. The differences in Cq values between the various studies may be based on differences in protocol, laboratories and instrumentation used. Additionally, the population of *Culicoides* vectors tested may differ between geographical regions and between individuals in terms of susceptibility, transmission of the virus and host preferences (Lord *et al.*, 1997).

An interesting phenomenon is that the difference between whole samples and dissected samples was not significant. For example, the mean Cq values of a pool size of 50 from this study and a pool size of 49 of abdomen samples only differed by approximately 0.5 units. This may prove important for the improvement and development of the methodology used in this study for prospective research or diagnostic assays, as this phenomenon questions the need for dissection, especially due to the fact that it is a slow process. Scheffer *et al.* (2012)

proposed the LOD at a pool size of 200 midges, where one individual is infected. Although the same was found in this study, it does not seem an accurate or reproducible LOD.

Table 2.3: Summary of the mean threshold cycle (Cq) values of different pool sizes for comparison between studies. This study is included and demarcated by the grey rows.

Pool size	Mean Cq value	Reference	Midge
1	30.75	This study	Whole
10	31.12	This study	Whole
25	32.30	This study	Whole
44	38.25	Scheffer *et al.*, 2012	Dissected
47	35.83	Scheffer *et al.*, 2011	Head
47	34.67	Scheffer *et al.*, 2011	Abdomen
49	36.74	Scheffer *et al.*, 2011	Head
49	32.52	Scheffer *et al.*, 2011	Abdomen
50	33.72	This study	Whole
66	32.84	Scheffer *et al.*, 2012	Dissected
76	36.20	Scheffer *et al.*, 2012	Dissected
80	27.63	Scheffer *et al.*, 2012	Dissected
93	37.28	Scheffer *et al.*, 2012	Dissected
100	32.92	This study	Whole
115	37.52	Scheffer *et al.*, 2012	Dissected
134	37.60	Scheffer *et al.*, 2012	Dissected
163	37.41	Scheffer *et al.*, 2012	Dissected
179	37.11	Scheffer *et al.*, 2012	Dissected
200	35.78	This study	Whole
200	33.43	Scheffer *et al.*, 2012	Dissected
228	29.25	Scheffer *et al.*, 2012	Dissected
292	33.39	Scheffer *et al.*, 2012	Dissected

Although limited studies are available where the *C. imicola* vector was directly assayed for the presence of AHSV, the above studies prove that it is a feasible approach to epidemiological studies. By using the vector itself, time consuming processes, such as virus isolation, become unnecessary. Diagnosis in vector samples therefore do not require the need for high-security laboratories, and the transport and storage of vector samples do not pose an issue for viral RNA isolation and the PCR process (Sailleau *et al.*, 1997). Diagnosis in vector samples does not pose the same problems as virus isolation from host samples. Host samples are sensitive to travelling and storage conditions (Sailleau *et al.*, 1997). Assays of vector samples can be done before the actual death of an infected host. RT-qPCR can successfully be applied to a variety of

pool sizes and detection in vectors is possible with different instruments, reagents and protocols. Further development of this technique is, however, still required. There is an increasing amount of data suggesting that other *Culicoides* species may be capable vectors of AHSV (Venter *et al.*, 2000). A pool screening technique can also be used for the identification of other possible vector species.

CHAPTER 3: APPLICATION OF RT-qPCR FOR THE DETECTION OF AHSV IN NAMIBIA

3.1 Introduction

Namibia (previously known as South-West Africa) is one of the least densely populated countries in the world with a total area of 824 292 km^2 and approximately 2.1 million inhabitants. It is located along the south Atlantic coast and is bordered by Angola, Zambia, Botswana and South Africa (Government of Namibia, 2014). Namibia is divided into 14 regions (Fig 3.1) (Government of Namibia, 2014). The country is known for the Namib Desert and arid environment. Main economic activities include tourism, mining and agriculture (Humavindu & Stage, 2013).

Figure 3.1: Map of Namibia. The 14 regions are indicated in orange. (Central Bureau of Statistics, 2009).

AHS is endemic in sub-Saharan Africa and occurs seasonally in late summer and early autumn in southern Africa (Coetzer & Gurthrie, 2004). Namibia has an arid climate that was not expected to sustain AHS presence (Becker, 2012). The horse industry in Namibia comprises approximately 61 902 horses (Directorate of Veterinary Services, 2000) and AHS outbreaks

impact negatively on the equestrian industry of this country (Molini *et al.*, 2015; Scacchia *et al.*, 2015). Namibia makes use of the OBP AHS vaccine for horses. A social survey in Namibia and South Africa found that 83% of 341 respondents vaccinated their horses with the OBP vaccine (Liebenberg-Weyers, 2015). Namibian veterinarians have emphasised the need for a monovalent AHS vaccine, targeted towards the serotypes present in Namibia (Caporale *et al.*, 2009). Additionally, the immune status of horses in Namibia is possibly variable, since vaccination in Namibia is not compulsory and inoculation of horses younger than 12 months of age should occur in three intervals (at 6, 9 and 12 months) and annually thereafter (Scacchia *et al.*, 2015).

Several AHSV serotypes have recently been isolated from blood and organ samples of horses in Namibia (Scacchia *et al.*, 2009; Scacchia *et al.*, 2015). These serotypes are present in central Namibia and samples originated from areas surrounding Windhoek, Okahandja, Omitara, Gobabis and Mariental (Scacchia *et al.*, 2009; Scacchia *et al.*, 2015) (Table 3.1), with most of the recorded and confirmed cases in being the pulmonary form of AHSV (Scacchia *et al.*, 2009). These cases also included the deaths of horses vaccinated against AHS (Scacchia *et al.*, 2009).

Culicoides knowledge and expertise in Namibia is insufficient at present (Becker *et al.*, 2012) and data concerning disease and vector distribution are limited (Becker *et al.*, 2012). Furthermore, the prevalence of AHSV in *C. imicola* in endemic areas has not yet been determined quantitatively (Scheffer, 2011).

Goffredo *et al.* (2015) found the following species of *Culicoides* in Namibia: *C. imicola*, *C. leucostictus*, *C. macintoshi, C. nivosus, C. pycnostictus*, *C. ravus, C. schultzei*, *C. subschultzei* and *C. tropicalis*. Becker (2012) found the following species in the Khomas region with UV light trap catches: *C. bedfordi, C. brucei, C. cornutus*, *C. expectator*, *C. herero*, *C. imicola*, *C. kanagai, C. leucostictus*, *C. macintoshi, C. magnus, C. nivosus, C. olyslageri, C. pretoriensis, C. pynostictus*, *C. ravus*, *C. remerki, C. schultzei, C. similis, C. subschultzei*, *C. tropicalis*, *C. tuttifrutti*, #89, #90 and #94. Of these *Culicoides* species, seven (*C. bedfordi*, *C. brucei*, *C. expectator*, *C. imicola, C. leucostictus*, *C. magnus*, and *C. pycnostictus*) have been implicated as possible vectors of AHSV in oral susceptibility experiments (Bellis, 2013). *Culicoides imicola* was found to be the most abundant and widespread species in Namibia, specifically in sample areas in the Khomas, Erongo, Otjozondjupa and Omaheke regions (Goffredo *et al.*, 2015). Thus, this species is likely to play a role in AHSV transmission in Namibia (Goffredo *et al.*, 2015). Furthermore, AHS distribution is dependent on the abundance of *Culicoides* (Venter *et al.*, 2006). Becker *et al.* (2013) has found that *Culicoides* numbers are more abundant in areas surrounding homesteads, specifically in the Khomas region. Homesteads therefore present a concentrated risk for the presence of AHSV vectors in these areas due to the availability of water around homesteads, especially during dry winter months (Becker *et al.*, 2013).

The use of RT-qPCR has been used for the purpose of determining presence of AHSV in Namibia (Goffredo *et al.*, 2015). Goffredo *et al.* (2015) sampled *Culicoides* from the Khomas, Erongo, Otjozondjupa and Omaheke regions. Only *C. imicola* from the Erongo region tested positive for AHSV. Pools were made up of either 50 or 100 individuals and the Cq value range for positive pools were shown to be between 29 and 39. Positive results were not obtained for the Khomas and Otjozondjupa regions (Molini *et al.*, 2015). Nevertheless, these sites are considered medium and high AHS incidence sites (Liebenberg *et al.*, 2015).

AHS has spread beyond endemic areas and has survived in these locations, indicating that the area theoretically appropriate for the transmission of the disease is substantially bigger than where it presently occurs (Wilson *et al.*, 2009). Thus, there is a need to sample and test *C. imicola* for the presence of AHSV in areas of low, medium and high AHS incidence, to evaluate the movement and persistence of the virus. There is a further need for epidemiological studies not only within the borders of endemic areas, but also areas that are at risk. AHS incidence and distribution is dependent on the presence of the vector, susceptible hosts and the virus (Venter *et al.*, 2006).

The aim of this chapter was the application of the RT-qPCR for the detection of AHSV in field-collected *C. imicola* pools in Namibia.

The specific objectives included the following:

- Determining the presence, abundance and diversity of *Culicoides* species in selected sites in Namibia (forming part of a larger AHSV and *Culicoides* study in Namibia).
- Applying RT-qPCR on field-collected *C. imicola* in a gradient of AHS-incidence sites in Namibia to detect AHSV.
- Comparing the presence of AHSV between different incidence sites.

Table 3.1: African horse sickness (AHS) reported cases and serotypes in Namibia (2006–2013). Table constructed from data from Scacchia *et al*. 2009 and Scacchia *et al*. 2015. RT-qPCR: real-time quantitative reverse transcription polymerase chain reaction.

Year	Area	Region	Diagnosis	Serotype	Notes
2008	Okahandja district	Otjozondjupa	RT-qPCR AHS positive only	-	Zebras present. AHS serotypes present include 1, 4, 6 and 9 (Scacchia *et al*., 2015)
2008	Okahandja district	Otjozondjupa	RT-qPCR & virus isolation	1	Zebras present. AHS serotypes present include 1, 4, 6 and 9 (Scacchia *et al*., 2015)
2008	Mariental district	Hardap	RT-qPCR & virus isolation	9	Borders the !Karas region. AHS Serotype 9 identified here (Scacchia *et al*., 2015)
2008	Between Windhoek and Gobabis districts	Omaheke	RT-qPCR & virus isolation	2	Vaccinated horse and animal died. Borders the Khomas and Otjozondjupa regions.
2008	Windhoek surrounds	Khomas	RT-qPCR AHS positive only	-	Horse vaccinated 7 months before and recovered. AHS serotypes identified include 1, 2, 4, 6, 8 and 9 (Scacchia *et al*., 2015)
2008	Okahandja district	Otjozondjupa	RT-qPCR AHS positive only	-	Vaccinated and recovered. AHS serotypes present include 1, 4, 6 and 9 (Scacchia *et al*., 2015)
2008	Swakopmund district	Erongo	RT-qPCR & virus isolation	2	Vaccinated and animal recovered. Borders Khomas and Otjozondjupa. AHS serotype 2 identified (Scacchia *et al*., 2015)
2006	Okahandja district	Otjozondjupa	RT-qPCR & virus isolation	4	Zebras present. AHS serotypes present include 1, 4, 6 and 9 (Scacchia *et al*., 2015)
2006	Gobabis district	Omaheke	RT-qPCR & Virus isolation	2	History of regular vaccination and animal died (Scacchia *et al*., 2009). Borders Khomas and Otjozondjupa. AHS serotypes identified here include 2, 6 and 8 (Scacchia *et al*., 2015)

3.2. Materials and Methods

3.2.1. Site selection and description

3.2.1.1. Namibian faunal and floral aspects related to the study and study sites

This study forms part of a larger study investigating the various factors (environmental and anthropogenic) involved in the distribution of AHSV in Namibia. In a multidisciplinary assessment of the distribution of AHS in Namibia the factors affecting the distribution of AHSV in this country have previously been investigated by Liebenberg *et al.* (2015). Site locations were chosen accordingly. A high, medium and low AHS incidence level site was chosen for the collection of *C. imicola* and the subsequent assay of collections. One of the traps on each site were allocated specifically for detection of AHSV in *C. imicola*. Three other traps per site were used to determine *Culicoides* species diversity and abundance.

Namibia has a unique biodiversity, with many endemic species. The plant and animal life are adapted to the arid environment and irregular rainfall (Mendelsohn *et al.*, 2010). The incidence level of AHSV in Namibia has been determined (Liebenberg *et al.*, 2015) and *Culicoides* collection sites were based on low, medium and high incidence of AHS. Aus (!Karas region) is a low AHS incidence site, Windhoek (Khomas region) is a medium AHS incidence site and Okahandja (Otjozondjupa region) is a high AHS incidence site. Table 3.2 shows the floral and faunal characteristics for these regions. Both the Khomas and Otjozondjupa regions have acacia tree and shrub savanna biomes (Mendelsohn *et al.*, 2010). !Karas' biome is a mixture between Nama Karoo, Namib Desert and Succulent Karoo (Mendselsohn *et al.*, 2010).

Zebras (mountain and plains zebra) are not found in high densities in these regions (Table 3.2). The zebra is considered the natural reservoir host of AHSV (Barnard, 1993). Antibodies for AHSV have also been found in zebras in the Khomas region (Becker, 2012). One of the sister species in the *C. imicola* complex, *C. kwagga* (#107), is also associated with the plains zebra and has the potential to be a vector for AHSV (Meiswinkel, 1995). AHSV antibodies have also been found in the African elephant (Lubroth, 1992) and another member of the *C. imicola* complex, *C. loxodonta* (Meiswinkel, 1992), is associated with the African elephant (Meiswinkel & Braack, 1994). This species may play a role in the cycling of orbiviruses (Meiswinkel, 1992). African carnivores, especially free-ranging carnivores, have been shown to carry several AHSV serotypes (Alexander *et al.*, 1995). Cheetahs especially are found in relatively high densities in the selected study regions.

Culicoides imicola, can be extremely abundant and widespread or totally absent in the presence of livestock (Meiswinkel, 1997, 1998) and the species has been shown to feed on horses, cattle,

pigs, sheep and goats (Meiswinkel *et al.*, 2004a; Nevill *et al.*, 1992). (The domestic animal densities are shown in Table 3.2.) The farming of livestock creates larval habitats for various *Culicoides* species through irrigation practices (Nevill *et al.*, 1992). There is an increased risk for AHSV transmission where horses and cattle occur close vicinity. This is due to increased numbers of *C. imicola* sister species, *C. bolitinos*, in these areas (Fall *et al.*, 2015) and to the immature stages of *C. bolitinos being* associated with the dung of herbivores such as cattle (Meiswinkel *et al.*, 2004a). The *Culicoides* species usually associated with livestock in southern Africa include *C. bedfordi, C. bolitinos, C. engubandei, C. fulvithorax*, *C. gulbenkiani*, *C. imicola, C. leucostictus*, *C. magnus*, *C. neavei*, *C. pycnostictus*, *C. schultzei*, *C. similis*, *C. tropicalis*, and *C. zuluensis* (Meiswinkel *et al.*, 2004a). *Culicoides* do not only feed on susceptible host species (Nevill, 1978). The impact of non-susceptible hosts, such as those mentioned above, in the transmission of arthropod-borne diseases is, therefore, an essential question in epidemiological research (Lo Iacono *et al.*, 2014). The risk of spread of viruses is often associated with feeding preferences and vector abundance (Lo Iacono *et al.*, 2014).

3.2.1.2. Namibian climate

Namibia is located along the southern coast of Africa and lies in the southern boundary of the tropics (Mendelsohn *et al.*, 2010). Drought is a common occurrence and there is a wide variation in annual rainfall across the country. A single wet season, with rainfalls predominantly between November and March, occurs. Some parts of Namibia, including the southwest of the country, occasionally receive winter rainfall (Sweet & Burke, 2000). Climatic conditions for the regions of interest are presented in Table 3.3.

Environmental conditions is one of the risk factors for AHSV outbreaks; other risk factors include *Culicoides* behaviour, vector abundance and the presence of non-susceptible hosts (Lo Iacono *et al.*, 2014). Rainfall and humidity have been shown to be the main drivers of AHSV outbreaks in Namibia (Liebenberg *et al.*, 2015). Thermal limits also play a role in the abundance and distribution of *C. imicola* (Verhoef *et al.*, 2014). All three regions have average maximum temperatures that are suitable for the optimum transmission of AHSV (Table 3.3). The average minimum temperatures in these regions are below that which is required for the overwintering of *C. imicola* (Mellor *et al.*, 2000). The average annual rainfall of the Khomas and Otjozondjupa regions falls within the range that permits the presence of *C. imicola* (Meiswinkel *et al.*, 2004a). Both low humidity and low temperatures, and high temperature and high humidity are not suitable for *Culicoides* survival (Wittman *et al.*, 2002). The average annual temperatures of the three Namibian regions and the relative humidity during the most humid months theoretically support AHS incidence.

Culicoides imicola will not is not present throughout the year in areas where frost occurs (Venter *et al*., 1997). Each of the selected regions experiences frost during the winter. However, *Culicoides* have been found in relatively high abundance in winter in the Khomas region and there is possible evidence of overwintering of AHSV in this region (Becker *et al*., 2012). Anthropogenic activity may be the mechanism for this survival (Becker *et al*., 2013).

Table 3.2: Namibian faunal and floral characteristics for the Khomas, Otjozondjupa and !Karas regions. Table constructed from Mendelsohn, 2006, Mendelsohn *et al*. 2010 and additional resources.

Faunal/floral aspects	Khomas (Windhoek area)	Otjozondjupa (Okahandja area)	!Karas (Aus area)	Notes
Biome	Acacia tree and shrub savanna		Mixture of Nama Karoo, Namib Desert and Succulent Karoo	-
Biome subdivision	Highland shrubland	Thornbush shrubland	Desert dwarf shurb transition, succulent steppe and southern desert respectively	-
Plant production	Very high	High/very high	Low/very low	*Culicoides imicola* adult populations occur in areas that are more sparsely vegetated and the species occurs in areas with low normalised difference vegetation index values (Conte *et al*., 2007).
Overall terrestrial diversity	High	High	Low	-
Grazing availability	Good	Good	Bad	-
Plant diversity (no. of species)	400–500	400–500	± 500	-
Mammal diversity (no. of species)	76–90	76–90	46–60	-
Density of large herbivores	Medium-high	Medium-high	Low	-

Springbok density	Low-medium	Medium-high	Medium	Potential wild host of haematophagous insects (Oshaghi *et al*., 2006).
Mountain zebra density	Low-medium	Low-medium	None	Considered the natural vertebrate host and reservoir of African horse sickness virus (AHSV) (Mellor & Hamblin, 2000; Wilson *et al*., 2009). Zebra in Khomas areas with AHSV antibodies (Becker, 2012).
Burchell's zebra	Low	Low	Low	Reintroduced to the south of Namibia (Mendelsohn *et al*., 2010).
Elephant density	None	None	None	AHSV antibodies present (Lubroth, 1992).
Gemsbok density	Medium-high	Medium-high	Medium	Potential wild host of haematophagous insects (Oshaghi *et al*., 2006).
No. of large carnivore species	4	4	5	-
Cheetah	High	High	High	AHSV antibodies present for serotype 7 (Alexander *et al*., 1995).
Spotted hyena	None	None	Medium	AHSV antibodies present for serotypes 1, 4, 6 and 7 – East African sample (Alexander *et al*., 1995).

Table 3.3: Namibian climate variables for the Khomas, Otjozondjupa and !Karas regions. Table constructed from the Atlas of Namibia (Mendelsohn *et al.*, 2010) and additional resources.

Climatic variables	**Khomas (Windhoek area)**	**Otjozondjupa (Okahandja area)**	**!Karas (Aus area)**	**Notes**
Hours sunshine per day	8–9	8–9	8–9	*Culicoides imicola* occurs in areas where they are exposed to full sunlight (Conte *et al.*, 2007).
Average annual temperature (°C)	18–20	20–22	18–20	African horse sickness virus (AHSV) infection is temperature dependent (Mullens *et al.*, 1995; Wellby *et al.*, 1996). *Culicoides* activity is associated with temperature (Blackwell, 1997).
Average maximum temperature (°C)	28–30	32–34	28–34	Increased temperature causes elevated virogenesis and transmission (Mullens *et al.*, 1995; Wellby *et al.*, 1996). *Culicoides* adults survival lower (Wellby *et al.*, 1996) Optimum temperature for AHSV transmission is 28–30°C (Wittman *et al.*, 2002).
Average minimum temperature (°C)	4–6	4–6	4–6	Decrease in temperature causes a decline in infection and virogenesis (Mullens *et al.*, 1995; Wellby *et al.*, 1996). Distribution is highly effected by low temperatures (Verhoef *et al.*, 2014).
Days of frost per year	10–20	5–10	5–10	*C. imicola* present throughout the year in frost-free areas (Venter *et al.*, 1997).
Average annual rainfall (mm)	300–350	300–400	50–100	*Culicoides* abundance is likely to be related to rainfall (Mellor *et al.*, 2000). Above-average rainfall will increase *C. imicola* numbers (Meiswinkel *et al.*, 2004a). *C. imicola* is present in areas where the annual rainfall is between 300–700 mm (Meiswinkel *et al.*, 2004a).
Low total rainfall (mm) (Indication of drought)	150–200	150–200	<25	Virus transmission is promoted by high temperatures during drought through vector replication (Sinclair, 2007; Gordon *et al.*,

				2013). AHSV epidemics occur in intervals of drought followed by heavy rains brought about by El Niño/Southern Oscillations , which causes an increase in vector numbers (Baylis *et al*., 1999).
Relative humidity (%) (In most humid months)	70–80	70–80	50–60	High humidity and high temperatures are unfavourable for the survival of *Culicoides* (Wittman *et al*., 2002).
Relative humidity (%) (In least humid months)	10–20	10–20	10–20	Low humidity and low temperatures are unfavourable for the survival of *Culicoides* (Wittman *et al*., 2002).

3.2.1.3. Okahandja (Otjozondjupa region)

The site chosen in this region is a horse farm, which falls under a high-incidence area of AHS (Liebenberg *et al.*, 2015). According to Molini *et al.* (2015), the Okahandja region had between six and ten AHS cases between 2006 and 2013. The farm is located on the outskirts of the town Okahandja. AHS has previously been reported on this farm, with fatalities of horses. There were approximately 25 horses on the farm, which were stabled in a semi-open warehouse. Other animals on and surrounding the farm included camels, oryx, honey badgers, cattle, goats and zebras. Horses were vaccinated annually with the OBP vaccine. There are no permanent bodies of water, but an ephemeral river runs through the farm. Irrigation practices on the farm were limited to gardening and lucerne propagation. The trap used for virus identification (Trap O1) was set up as close as possible to the horse stable (Fig 3.2) to ensure maximum collection of blood-fed *Culicoides* females in the vicinity of the host. The trap was located at the following GPS coordinates (S21°58.363', E016°55.330') at an altitude of 1 348 m above sea level. Traps labelled O2–O4 were used for *Culicoides* identification and abundance.

Figure 3.2: Okahandja, high incidence site for African horse sickness, for the collection of *Culicoides* to determine the presence of African horse sickness virus. Trap O1 was used for real-time quantitative reverse transcription polymerase chain reaction assays (Liebenberg-Weyers, 2015).

3.2.1.4. Windhoek (Khomas region)

The medium incidence site (Liebenberg *et al.*, 2015) was located on a horse stud outside of Windhoek in the Khomas region. According to Molini *et al.* (2015), the Windhoek district reported between 11 and 25 AHS cases between 2006 and 2013. This stud has struggled with AHS and has suffered great losses. There were about 25 stabled horses, housed in open paddocks, and a small number of free-roaming horses on the farm. Other animals on and surrounding the farm included cattle, goats and chicken. Zebras were not found nearby. The OBP vaccine was administrated annually during the winter months. There are no permanent bodies of water present on the farm, but there is an ephemeral river and irrigation practices. The trap (W1) was set up as close as possible to the horse stable (Fig 3.3), to ensure optimum collection of blood-fed *Culicoides* females in the vicinity of the host. The trap was labelled W1 (for virus identification) and located at the following GPS coordinates (S22°26.647', E017°35.628') at an altitude of 1 627 m above sea level. Traps labelled W2–W4 were used for *Culicoides* identification and abundance.

Figure 3.3: Windhoek, medium incidence site for African horse sickness, for the collection of *Culicoides* to determine the presence of African horse sickness virus. Trap W1 was used for real-time quantitative reverse transcription polymerase chain reaction assays (Liebenberg-Weyers, 2015)

3.2.1.5. Aus (!Karas region)

The site, located in the !Karas region,is a low AHS incidence area (Liebenberg *et al.*, 2015) and had no record of AHS in the last 100 years. The feral horses of Namibia occur in this area. They

range a large area due to the sparse availability of grazing and water and animals such as goats and sheep were present intermittently in this area. Generally, horses in this area have not been vaccinated, but there is a record of a single horse being vaccinated in 2013 for competition purposes. There are some limited irrigation practices on this site. The trap (A1) was set up as close as possible to the water source and since horses are not stabled, this area was presumed to be the best breeding habitat for *Culicoides* (Fig 3.4). The trap labelled A1 (used for virus identification) was located at (S26°39.375', E016°14.840') at an altitude of 1 408 m above sea level. Traps labelled A2–A4 were used for *Culicoides* identification and abundance.

Figure 3.4: Aus, low incidence site for African horse sickness, for the collection of *Culicoides* to determine presence of African horse sickness virus. Trap A1 was used for real-time quantitative reverse transcription polymerase chain reaction assays (Liebenberg-Weyers, 2015).

3.2.2. *Culicoides* collection, sorting and identification

Culicoides adults were collected weekly over a 20-week period from January to May in both 2013 and 2014. This period coincided with the AHS season. Collections were made on all three sites using the Onderstepoort 220 V suction UV-light trap as described previously (Venter *et al.*, 1998). Traps were operated from sunset to sunrise, when biting midges are most active (Meiswinkel *et al.*, 2004a). Collections were made in 30% ethylene glycol to ensure that collections were well preserved. Weekly collections were emptied (on the same day for each site), rinsed and preserved in 70% ethanol.

Culicoides wing pattern was used for identification. Specimens where only identified up to the *C. imicola* complex to ensure increased accuracy for virus identification. A subsampling procedure was followed as described by Van Ark and Meiswinkel (1992). *Culicoides* species from each weekly sample were separated from other insects under a stereomicroscope and identified morphologically using the wing picture atlas of Afrotropical *Culicoides* (Meiswinkel, 1996). *Culicoides imicola* complex samples were separated into different categories, based on parity according to the age-grading technique described by Dyce (1969).

Traps O2–O4, W2–W4 and A2–A4 were used for species identification at the respective study sites. *Culicoides* identification was done by a specialist (Karien Labuschagne) from ARC-Onderstepoort. Identification between subgenera had to be performed by a specialist, due to the difficulty in discerning between *Culicoides* subgenera based on wing patterns (Nevill *et al*., 2007).

3.2.3. Absence of presence of AHSV

Although collections were made weekly, two weeks' collections were grouped together. Pooling was done due to the expense of the viral RNA extraction and RT-qPCR reagents. The optimum pool size range for the detection of AHSV in *C. imicola* pools was determined in Chapter 2. For the purpose of the application of the RT-qPCR on Namibia field *Culicoides*, a pool size of 30 was chosen. *Culicoides* pools were assayed according to the procedure as described in Chapter 2. Samples were divided into triplicate pools of 30 blood-fed female *C. imicola* individuals. Blood-fed females were used to increase the chances of detecting an infected individual in a field situation. Where 90 (three pools of 30) blood-fed female *C. imicola* were not available, the available specimens were assayed. Pools of 30 individuals were used based on the guideline in Chapter 2, below 50 individuals per pool allows for detection with satisfactory reliability and efficiency. Furthermore, limited number of *Culicoides* were available for processing and thus the use of this number of individuals.

Fully blood-engorged *C. imicola* complex females were sorted and stored in micro-centrifuge tubes at -4°C until the assay was performed. Based on abdominal pigmentation (Dyce, 1969), groups of unfed nulliparous *C. imicola* were identified and stored in pools of 30 for the negative control groups of the assay. The positive controls and NTC were the same as those used in Chapter 2. In field conditions the number of days after ingestion of infected blood by the *Culicoides* female is unknown (Veronesi, 2014) and no information on the feeding history of individuals is available. Thus, there may be differences in the virus titre volume in samples.

3.3. Results and Discussion

3.3.1. *Culicoides* species composition and abundance

The *Culicoides* species identification section forms part of a larger research project and, therefore, some results will correspond to the study of Liebenberg-Weyers (2015). Table 3.4 shows the *Culicoides* sample identification for each site. The table takes into account overall presence and numbers of *Culicoides* species at the three sites. Dark grey areas indicate principal AHSV vectors (*C. imicola* and *C. bolitinos*), while lighter grey shading indicates other reported AHSV vectors (Bellis, 2013). Other species implicated as vectors of AHSV were described in Chapter 1.

There were 397 collections made over the survey period. Of these collections, 102 (from trap 1, i.e. O1, W1 and A1 respectively) were used in the RT-qPCR for the detection of AHSV. A total of 295 collections were processed for total *Culicoides* information. Seventy-five percent of the 295 collections were used for detailed identification of *Culicoides* species.

At the high AHS incidence site (Okahandja) 82 collections were identified. A total of 47 343 *Culicoides* were collected. Okahandja had the highest number of *Culicoides* species and *C. imicola* as compared to Aus and Windhoek. A total of 48 species were identified from all three sites, most of which were present in Okahandja (41). The two main vectors for AHSV, *C. imicola* and *C. bolitinos,* were both present on this site. *Culicoides imicola* comprised 42.7% of the total number of *Culicoides* identified. Various species associated with livestock were present at this site (*C. bedfordi*, *C. bolitinos, C. imicola*, *C. leucostictus*, *C. neavei*, *C. pycnostictus*, *C. schultzei*, *C. similis* and *C. tropicalis*) (Meiswinkel *et al*., 2004a). The presence of these species may be attributed to the presence of other animals on the farm, especially livestock such as cattle and goat. Although below 0.1% of the total *Culicoides* collected, both *C. kwagga* (#107) and *C. loxodontis* were present at Okahandja, which are associated with zebras (Meiswinkel, 1995) and elephants (Meiswinkel, 1992) respectively. The presence of *C. kwagga* may be explained by the presence of zebras on the farm, which are considered the reservoir host of AHSV (Mellor & Hamblin, 2004). No known elephants occur in the area. Both these *C. imicola* sister species may have a potential role as AHSV vectors (Meiswinkel, 1992, 1995.) Other important species present at the Okahandja site included those implicated as possible AHSV vectors: *C. expectator* (17.9%), *C. leucostictus* (3.3%), *C. pycnostictus* (2.3%), *C. enderleini* (1.3%), *C. bedfordi* (0.3%) and *C. brucei* (<0.1%) (Bellis, 2013).

Table 3.4: *Culicoides* collected and identified at Aus, Windhoek and Okahandja in Namibia. The table shows the number of collections, the number of species and the total collection each species makes up, for each site (Liebenberg-Weyers, 2015). Dark grey areas indicate principal African horse sickness virus (AHSV) vectors while light grey shading indicates other reported AHSV vectors (Bellis, 2013).

Site	Aus	Okahandja	Windhoek	Total
No. of collections identified	83	82	59	
No. of species	21	41	36	48
Species	**% of site total**	**% of site total**	**% of site total**	**% of grand total**
C. imicola	6.8	42.7	12.7	29.9
C. subschultzei		14.7	28.4	16.6
C. expectator	*	17.9	8.9	13.2
C. ravus	45.9	4.4	11.4	11.5
C. herero	31.5	3.0	12.5	9.2
C. tropicalis	*	0.6	11.9	3.6
C. pycnostictus	0.1	2.3	5.2	2.8
C. leucostictus	0.2	3.3	1.8	2.5
C. nivosus	0.7	2.0	1.9	1.8
C. schultzei	*	2.1	0.4	1.4
C. pretoriensis	0.1	1.3	1.3	1.2
C. similis	5.3	0.4	0.7	1.1
C. enderleini	0.1	1.3	0.1	0.8
C. bedfordi	3.8	0.3	0.3	0.8
#89	4.9	*	*	0.6
#61		0.4	0.9	0.5
C. tuttifrutti (#30)	0.1	0.7	0.1	0.5
Accraensis group		0.6	0.1	0.4
C. punctithorax	0.1	0.5	*	0.3
C. tororoensis			1.0	0.3
C. albopunctatus		0.3	0.1	0.2
C. bolitinos		0.3	0.1	0.2
C. coarctatus		0.2	*	0.1
C. eriodendroni		0.1	*	0.1
C. neavei		0.1	*	0.1
C. brucei	0.2	*	*	0.1
#54 (d/f)		0.1	*	*
Nigripennis group		0.1	*	*
C. cornutus		*	0.1	*
C. macintoshi	0.2		*	*
#94	*	*		*
C. distinctipennis		*		*
#62			*	*
C. miombo		*	*	*

C. kanagai		*		*
C. rhizophorensis		*		*
#50		*	*	*
#54 (p/f)		*		*
#33	*		*	*
#107		*		*
C. glabripennis			*	*
C. loxodontis		*		*
C. nevilli		*		*
C. olyslageri		*		*
C. ovalis		*		*
C. dekeyseri	0.1			*
C. trifasciellus			*	*
#69		*		*
Total	**9980**	**47343**	**21819**	**79142.4**

**Culicoides* species comprising less than 0.1% if the site and total collection.

Collections from the medium AHS incidence site, Windhoek, had fewer *Culicoides* (21 819) than Okahandja. Windhoek also had a lower number of species than Okahandja, with 36 different *Culicoides* species. The species with the highest number of individuals present at this site was *C. subschultzei*, comprising 28.4% of the total *Culicoides* identified here. However, this species is not a suspected or known vector of AHSV. The principle vector, *C. imicola* was present (12.7%), but in lower numbers than at the high AHS incidence site. *Culicoides* species present at Windhoek were similar to those found by Becker (2012) and 21 of the species correlated with those collected by Becker (2012). These species included *C. bedfordi, C. brucei*, *C. cornutus*, *C. expectator*, *C. herero*, *C. imicola*, *C. kanagai, C. leucostictus, C. macintoshi*, *C. nivosus, C. olyslageri, C. pretoriensis, C. pycnostictus, C. ravus*, *C. schultzei, C. similis, C. subschultzei*, *C. tropicalis*, *C. tuttifrutti*, #89 and #94 (Becker, 2012). The same species associated with livestock were found both at the Okahandja and the Windhoek sites. Livestock on the latter farm included chicken, cattle and goat, which have been shown to be hosts for *C. imicola* (Meiswinkel *et al*., 2004a). Other AHSV vectors found at this site included *C. expectator* (8.9%), *C. pycnostictus* (5.2%), *C. leucostictus* (1.8%), *C. bedfordi* (0.3%), *C. bolitinos* (0.1%) and *C. brucei* (less than 0.1%) (Bellis, 2013).

Aus, the low AHS incidence site, had a total of 83 collections and 21 *Culicoides* species were identified. Aus collections had considerably fewer species and *Culicoides* species than the other two sites. *Culicoides imicola* made up 6.8% of the total *Culicoides* in Aus. The most abundant species in Aus was *C. ravus* (45.9%) and the second-most abundant species was *C. herero* (31.5%). No information is available on these species' ability to transmit viruses (Meiswinkel, 1996). *Culicoides* species, similar to those found at Okahandja and Windhoek, associated with livestock were found at Aus, but occurred in lower numbers. *Culicoides bolitinos and C. neavei*

were not present at Aus as in the high and medium AHS incidence sites and *C. bolitinos* was not present, even though cattle were present on the farm. Other *Culicoides* implicated as AHSV vectors found at this site included *C. bedfordi* (3.8%), *C. brucei* (0.2%), *C. leucostictus* (0.2%), *C. enderleini* (0.1%), *C. pycnostictus* (0.1%) and *C. expectator* (less than 0.1 %) (Bellis, 2013).

A total of 48 species were identified, of which the principal vector, *C imicola,* was the most abundant overall. A total of 9 980 *Culicoides* were identified in Aus, 21 819 in Windhoek and 47 343 in Okahandja. Overall, a total of 79 142 *Culicoides* were identified. It is important to consider that UV-light suction light trap collections only represent an extremely small proportion (0.0001%) of the total *Culicoides* population (Venter *et al.*, 2009). The total number of *Culicoides* increased when moving from the low AHS incidence site to the high AHS incidence site. The same trend was noted with the total number of *Culicoides* species in each site, with 21 species collected in Aus (low AHS incidence), 36 species collected in Windhoek (medium AHS incidence) and 41 species collected in Okahandja (high AHS incidence).

Culicoides species found in this study corresponded to those found by Goffredo *et al.* (2015). Eight of the 12 *Culicoides* species implicated as AHSV vectors (Bellis, 2013) are found in Namibia based on the identification data. Figure 3.5 shows the percentage of implicated AHSV vectors at each site. Number of vector individuals were calculated for each site. Aus, the low AHS incidence site, had approximately 1 117 AHS vector individuals out of a total of 9 980 *Culicoides* individuals collected. Windhoek, the medium AHS incidence site, had roughly 6 349 AHS vector individuals out of a total of 21 819 *Culicoides* individuals. Okahandja, the high AHS incidence site, had approximately 32 240 vector individuals out of the total 47 343 *Culicoides* individuals collected. The percentage vector individuals increased from the low incidence site to the high incidence site (Fig 3.5).

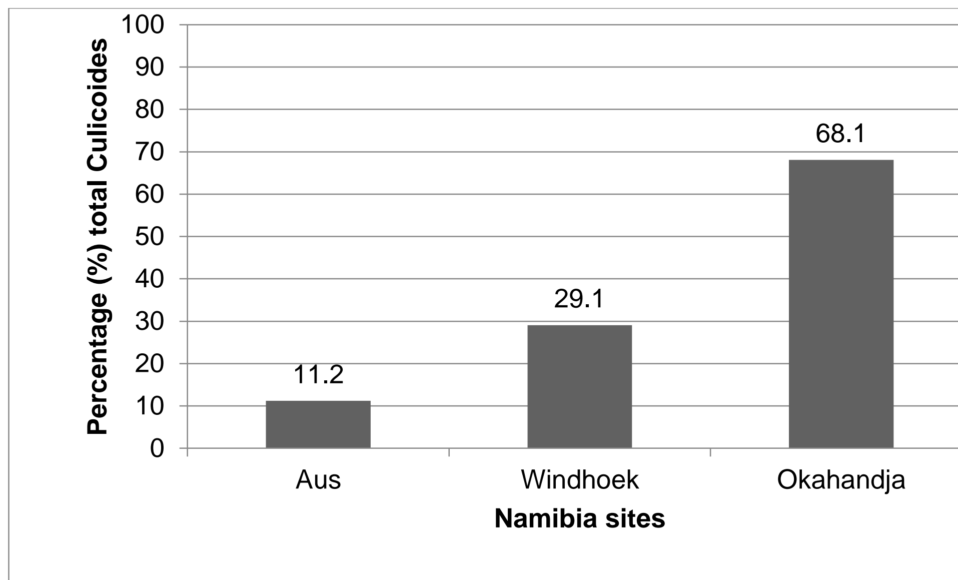

Figure 3.4: Bar graph indicating the percentage *Culicoides* that are implicated vectors for African horse sickness virus at each site

The total number of *Culicoides* collected from all the traps from each site grouped fortnightly for each year is given in Table 3.5. Total catches per trap were added and averages calculated. Traps with no collections were not taken into account, but collections without *Culicoides* present were taken into consideration.

In Okahandja, the high AHS incidence site, the highest number of *Culicoides* was found in week 9 and 10 (March) of 2013. The lowest numbers were found in week 19 and 20 (May) of the same year, at the end of the survey period. In 2013, Windhoek had the highest total number of *Culicoides* in weeks 7 and 8 (March) and in week 9 and 10 (March). No average was made for week 9 and 10 collection as no discernment could be made from which trap each sample came. Lowest numbers were recorded in week 15 and 16 (end of April and beginning of May) with 158 *Culicoides* collected. During the same year in Aus, the highest number of *Culicoides* was collected in week 1 and 2 (January). The lowest number *Culicoides* for this site was in week 19 and 20 (May), at the end of the survey period. The collections of week 13–16 were added due to a collection error by the assigned person.

In 2014, at the high AHS incidence site, Okahandja, the highest numbers were recorded during weeks 3 and 4 (end of January and beginning of February). The lowest *Culicoides* numbers were recorded in week 17 and 18 (May) with 137 individuals. In Windhoek, weeks 9 and 10 (March) yielded the highest numbers in 2014. The lowest *Culicoides* numbers were recorded in weeks 17 and 18 (May). The low AHS incidence site, Aus, had the highest number of total *Culicoides* during weeks 3 and 4 (end of January and beginning of February) of 2014, and weeks 19 and 20 (May) revealed the lowest numbers of *Culicoides*. There seems to have been a seasonal pattern of decline in *Culicoides* numbers, as the temperature also declines seasonally.

Table 3.5: The total number of *Culicoides* collected from all the traps from each site grouped fortnightly, for each year. The average number of *Culicoides* is given in brackets. Dark grey shading indicates weeks with the highest number of *Culicoides* and light grey shading indicates weeks with the lowest *Culicoides.*

2013	**Aus**	**Windhoek**	**Okahandja**
Week 1+2	11 716 (1 952.7)	5 551 (925.2)	7 834 (1 305.7)
Week 3+4	3 314 (552.3)	568 (142)	6 463 (1 077.2)
Week 5+6	1 996 (332.7)	5 808 (968)	6 745 (1124.2)
Week 7+8	4 371 (728.5)	6 969 (1 161.5)	7 907 (1 317.9)
Week 9+10	4 824 (804)	11 115*	11 700 (1 950)
Week 11+12	186 (37.2)	6 324 (1 054)*	4 808 (801.3)
Week 13+14	1 004*	3 043 (1 014.3)	4 235 (705.8)
Week 15+16		158 (26.3)	7 586 (1 264.3)
Week 17+18	116 (19.3)	415 (69.2)	1 835 (305.8)
Week 19+20	76 (12.6)	2 057 (342.8)	1 811 (301.8)
2014	**Aus**	**Windhoek**	**Okahandja**
Week 1+2	1 313 (437.7)	874 (145.7)	2 546 (424.3)
Week 3+4	1 583 (263.8)	390 (130)	5 585 (930.8)
Week 5+6	561 (93.5)	1 189 (396.3)	4 312 (718.7)
Week 7+8	1 202 (240.4)	400 (100)	4 852 (808.7)
Week 9+10	259 (51.8)	1 325 (331.25)	1 592 (265.3)
Week 11+12	88 (14.6)	1 283 (320.75)	872 (174.4)
Week 13+14	56 (11.2)	1 050 (262.5)	573 (95.5)
Week 15+16	162 (32.4)	774*	419 (83.8)
Week 17+18	65 (13)	65 (32.5)	137 (22.8)
Week 19+20	38 (6.3)	742 (185.5)	437 (72.8)

Only *C. imicola* midges were assayed to determine the presence of AHSV at different sites. Nevertheless, the importance of other *Culicoides* species implicated as AHSV vectors should not be underestimated and should be further researched. Differences between sites may be caused by a wide array of factors. These may include climatic variables, such as rainfall and temperature differences between the three sites. The presence and abundance of host species may also differ between sites, leading to differences in numbers and species of *Culicoides*.

3.3.2. AHSV RT-qPCR assay

Table 3.6 shows the RT-qPCR presence data for AHS viral RNA in *C. imicola* at Aus, Windhoek and Okahandja for both 2013 and 2014.

Table 3.6: The African horse sickness virus (AHSV) real-time quantitative reverse transcription polymerase chain reaction results for each site and fortnight for 2013 and 2014. Shaded areas indicate fortnights during which positive results were obtained. -: No collection made or no *Culicoides imicola* (vector) in sample; X: negative for AHSV viral RNA; ✓: AHSV viral RNA detected.

2013	Aus	Windhoek	Okahandja
Week 1+2	✓	✓	x
Week 3+4	X	X	X
Week 5+6	X	X	X
Week 7+8	X	X	X
Week 9+10	X	✓	X
Week 11+12	X	X	✓
Week 13+14	X	X	X
Week 15+16	X	-	X
Week 17+18	X	X	X
Week 19+20	X	X	X
2014	**Aus**	**Windhoek**	**Okahandja**
Week 1+2	-	X	X
Week 3+4	-	X	X
Week 5+6	X	X	X
Week 7+8	-	X	✓
Week 9+10	X	✓	X
Week 11+12	X	✓	x
Week 13+14	X	X	X
Week 15+16	-	X	X
Week 17+18	-	X	X
Week 19+20	X	X	X

AHSV was detected in Okahandja, the high AHS incidence site, during weeks 11 and 12 (29/03 and 05/04) in 2013. Windhoek, the medium AHS incidence site, yielded positive results for weeks 1 and 2 (18/01and 25/01) and weeks 9 and 10 (15/03 and 22/03) of the same year. AHSV was detected in the low AHS incidence site, Aus, during weeks 1 and 2 (18/01 and 25/01) of 2013. Detection was evident during the first half of the survey period in January. In 2014 detection was also concentrated around the first half of the survey period, from the end of February to the beginning of April. The highest seasonal incidence occurred in summer and early autumn, between February and June (Coetzer & Gurthrie, 2004; Gordon *et al.*, 2013: Monaco *et al.*, 2011). These results were expected to correlate with the AHS season and the correlation is evident from the dates when positive results were obtained. These dates fall within the summer to early autumn timeframe. AHSV-positive results in 2014 were found for Okahandja from weeks 7 and 8 (28/02 and 07/03). Weeks 9 and 10 (14/03 and 21/03) and weeks 11 and 12 (28/03 and 04/03) revealed AHSV RNA for Windhoek in 2014. No positive

results were found for Aus in 2014. The results were obtained in accordance with the AHS season.

Table 3.7 shows the detailed results for all AHSV-positive samples. Three pools were made up of 30 *C. imicola* blood-fed females each. The table shows which of the three pools (A, B or C) yielded a positive AHSV result. It also shows how many of the three replicates of that specific pool yielded a positive result.

Table 3.7: Detailed real-time quantitative reverse transcription polymerase chain reaction results for all African horse sickness virus-positive results at Okahandja, Windhoek and Aus in Namibia for January to

Site	Year	Week	Collection dates	No. individuals per pool	Pool no.	Replicates positive (out of 3)	Average Cq values
A	2013	1+2	18 & 25/01	30	B	1	14.71
W		1+2	18 & 25/01		A	2	29.39
W		9+10	15 & 22/03		C	1	21.56
O		11+12	29/03 & 05/04		C	1	13.63
W	2014	9+10	14 & 21/03	30	C	1	38.53
W		11+12	28/03 & 04/04		A	2	29.92
W		11+12	28/03 & 04/04		B	3	35.03
O		7+8	28/02 & 07/03		C	3	30.00

May (2013 and 2014). A: Aus; W: Windhoek; O: Okahandja; Cq: threshold cycle.

The high AHS incidence site, Okahandja, revealed a positive result for the collection dates 29/03/2013 and 05/04/2013. Only one of the three replicates of pool C was positive for AHSV. The Cq value was low at 13.63. This indicates that the starting quantity of viral RNA was high. An AHS mortality was reported close to the dates during which a positive AHSV result was obtained. More positive results were expected since it is a high AHS incidence area and *C. imicola* was present in high numbers (Table 3.5). AHS has been problem on this farm over the last five years. Additionally, AHSV serotypes have been identified in Okahandja between 2006 and 2013 (Scacchia *et al.*, 2015).

Windhoek displayed two AHSV positive results for the survey period in 2013. For weeks 1 and 2 (18/01 and 25/01), two out of the three replicates from pool A yielded a positive result with a mean Cq value of 29.39. During weeks 9 and 10 (15/03 and 22/03) of the same year, only one replicate in pool C tested positive for AHSV. The mean Cq value was 21.56. AHS has been a problem on this site. The highest *Culicoides* numbers for Windhoek in 2013 were recorded during weeks 7 and 8 (01/03 and 08/03) and weeks 9 and 10 (15/03 and 22/03) (Table 3.5),

which corresponded to the weeks during which the second positive result was obtained. AHSV serotypes have been identified in Windhoek between 2006 and 2013 (Scacchia *et al.*, 2015).

Positive results for the low AHS incidence site, Aus, occurred between the collection dates of 18/01/2013 and 25/01/2013. The vaccination of a single horse occurred after these dates in which the AHSV RNA was detected. Only one of the three replicates of pool B was positive for AHSV and the Cq value (14.71) was very low. A low Cq value indicates a high starting number of target cDNA. The AHSV positive result for Aus was unexpected, especially since this is a low AHS incidence site and AHS has not been recorded here for the last 100 years. Additionally, *Culicoides* was found in very high numbers (Table 3.5) during these weeks. The highest number collected in Aus corresponded with the collection dates during which a positive AHSV result was obtained. A rainfall event was observed by the farmer, with associated flooding of the river on site. This could have had an effect on *Culicoides* reproduction and numbers. As animals aggregate at water holes in this site's arid location, the probability of AHSV in vectors could have increased due to exposure to AHSV hosts.

The Cq values for all AHSV positive results in 2013 across all sites were low. These Cq values were generally lower than the values obtained with pool size testing in Chapter 2. The first two weeks of the survey period revealed positive results in two sites. Successive detection occurred in weeks 9 and 10 (Windhoek) and weeks 11 and 12 (Okahandja). This may indicate certain periods of increased transmission of AHSV. Certain weather phenomena or the movement of hosts may be possible causes for simultaneous results in different sites. There seems to be a pattern between high *Culicoides* numbers and the occurrence of positive results.

The high AHS incidence site, Okahandja, had one positive pool for the presence of AHSV in 2014. Weeks 7 and 8 (28/02 and 07/03), during which this result was obtained, preceded the dates during which positive results were obtained in Windhoek. Previous testing of *C. imicola* for the presence of AHSV using RT-qPCR in Okahandja yielded negative results (Goffredo *et al.*, 2015).

Of all the sites, the medium AHS incidence site, Windhoek, had the most AHSV positive results in 2014. The positive results for weeks 9 and 10 (14/03 and 21/03) corresponded with the fortnightly grouping during which the most *Culicoides* was recorded (Table 3.5). Thus, these high numbers also preceded the two following weeks during which the second positive test result was obtained. Previous testing of *C. imicola* for the presence of AHSV using RT-qPCR in Windhoek yielded negative results. However, an AHSV-positive result was obtained using virus isolation for an April 2011 collection (Goffredo *et al.*, 2015). The positive results for Windhoek during weeks 11 and 12 (28/03 and 04/04) of 2014 coincided with the period (April 2011) during which AHSV-positive results were obtained via virus isolation by Goffredo *et al.* (2015). No positive result was obtained for the detection of AHSV in Aus in 2014. These results were

expected for Aus since this is the low AHS incidence site and *Culicoides* were less abundantly present compared to the other two sites.

The close proximity of positive pools and the high number of positive replicates in Windhoek and Okahandja may indicate a high possible AHS incidence period. Furthermore, AHSV serotypes have been identified from both Okahandja and Windhoek between 2006 and 2013 from host tissue samples (Scacchia *et al.*, 2015). Previous assays for the detection of AHSV in *Culicoides* midges for both Okahandja and Windhoek were negative (Goffredo *et al.*, 2015). There seems to be some relation between high *Culicoides* numbers and the dates during which AHSV were detected. The Cq values for 2014 were considerably higher than those of 2013. The variation in Cq value may be based upon different volumes of blood meals taken by female *C. imicola* or by the number of AHSV infected females detected by the RT-qPCR. All the results obtained during 2014 were within a six-week period, from week 7 to 12 (28/02 to 04/03). Some climatic phenomena may be responsible for virus detection during this period. Windhoek, the medium AHS incidence site, produced the most AHSV-positive results. This was unexpected. Additionally, recorded AHS cases for the Windhoek district was higher than for the Okahandja district from 2006 to 2013 (Molini *et al.*, 2015). Okahandja had the highest numbers of *Culicoides* vectors in total, but Windhoek had the highest number of positives, indicating another driver for AHS at this site.

The presence of AHSV in host tissue samples from the sites (Scacchia *et al.*, 2015) is a good indication that the virus will most likely also be present in the vector midges and was shown by the results above. Limited literature is available on the successful detection of AHSV in *C. imicola* pools using RT-qPCR in Okahandja, Windhoek and Aus. The results contribute to limited application of RT-qPCR in *Culicoides* for the detection of AHSV in Namibia.

CHAPTER 4: CONCLUSION

AHS is an arthropod-borne disease primarily affecting equids and is transmitted by *C. imicola* (Meiswinkel *et al.*, 2004a; Mellor *et al.*, 2000). This disease has a high mortality rate in susceptible horses and there is a risk for expansion beyond endemic ranges (OIE, 2014). Several studies have covered the use and optimisation of RT-qPCR for the detection of AHSV in host tissue samples (Aradaib *et al.*, 2006; Guthrie *et al.*, 2013; Quan *et al.*, 2010; Sailleau *et al.*, 1997). However, the application of RT-qPCR on vector midges is lacking. The importance of applying such a diagnostic technique to vector midges lies in the rapidity of results and information obtained before the death of hosts or an outbreak event occurs. The application of RT-qPCR in vector midges can be used to determine the presence of AHSV. Such information may be valuable where data are not available on AHSV presence and distribution. Additionally, no standard operating procedure is available for the processing of *C. imicola* using RT-qPCR assays and the LOD of AHSV in *C. imicola* pools is unknown. In order to detect AHSV in field-collected *C. imicola* complex pools in Namibia using RT-qPCR, four objectives were set. A concise conclusion on the outcome of the objectives is given below.

4.1. The determination of the sensitivity of the RT-qPCR methodology

Various parameters were used to determine the suitability (sensitivity) of the RT-qPCR method for the detection of AHSV. A linear relationship was found between Cq values and RNA concentration. The hypothetical LOD was found to be 10^1 copies of target cDNA. The efficiency was 108.3%. On the basis of these results it was shown that the specific methodology could successfully and accurately be applied for the detection of AHSV and the subsequent application of RT-qPCR to *C. imicola* complex pools.

4.2. The determination of the limit of detection of AHSV in *C. imicola* complex pools.

To determine the optimal pool size for the detection of a single infected *C. imicola* individual, different pool sizes were constructed and assayed; results were compared between pool sizes. Various experimental parameters were taken into account in determining the optimal pool size for AHSV detection, including Cq values, cost effectiveness, starting quantity of target nucleic acids, assay sensitivity, number of individuals available and time efficiency. From what was observed a guideline was provided on LOD rather than a specific pool size. For the purpose of this study, with the application of the RT-qPCR methodology, a pool size of below 50 was considered adequate for AHSV detection. An increase in pool size could increase the risk for false negatives and a dilution effect may be present.

The application of the RT-qPCR to *C. imicola* of various pool sizes has emphasised important considerations and limitations. There may be low virus titre present in a blood meal or a low volume blood meal may be taken by a female individual. It is also important to consider that not all midges in a population may be susceptible to AHSV infection and that some *Culicoides* may carry the virus below detectable levels (Scheffer *et al*., 2011). This may have an effect on the prevalence of positive results. Additionally, target molecules may not be distributed evenly amongst replicates, known as a Poisson distribution. A high number of replicates are required to eliminate this effect. However, other factors such as assay sensitivity, cost efficiency and the number of individuals available for processing also need to be considered. The difference between whole and dissected samples for AHSV detection assays is not noteworthy. This may be an important improvement of methodology, since the need for dissection is questioned, especially due to the fact that it is a time-consuming process.

4.3. The determination of the presence of AHSV in *C. imicola* using RT-qPCR in Namibia

The RT-qPCR methodology was applied to a field situation, with sites of different AHS incidences in Namibia. AHSV was present in all sites (low, medium and high AHS incidence) across the survey period in 2013. In 2014, however, AHSV was only present in the medium (Windhoek) and the high AHS incidence site (Okahandja). Thus the application of a pool screening assay for the detection of AHSV in *C. imicola* complex pools in a natural environment was successful. Additionally, *Culicoides* species and abundance were also determined. A total of 48 *Culicoides* species were identified across the sampling sites in Namibia. *Culicoides imicola*, the principal vector of AHSV (Meiswinkel *et al*., 2004a), was found to be the most abundant species. Other possible AHSV vector species were also found at the study sites. There could be an association between high *Culicoides* numbers found in traps and positive results obtained with the RT-qPCR assay; however, this does require more research.

4.4. The comparison of the prevalence of the virus in the vector collected at different sites in Namibia

A comparison of the prevalence of AHSV in *C. imicola* collected at three different sites was made to establish the relationship between incidence levels and actual AHSV presence in *C. imicola* pools. Windhoek, the medium AHS incidence site, yielded the highest number of positive results over the two-year survey period, followed by the high AHS incidence site, Okahandja. The low AHS incidence site, Aus, had the lowest number of positive results and AHS has not been a problem there during the last 100 years. However, a rainfall event was possibly linked to the presence of AHSV during weeks 1 and 2 of 2014. It was expected that

most positive results would be obtained from the high AHS incidence site. The results in Okahandja can be linked to an AHS mortality. *Culicoides* numbers also seem to have an effect on the presence of AHSV. Where high numbers of *Culicoides* were present, accompanying positive results were obtained, although *Culicoides* may not be the only factor responsible for presence of AHSV. There is a wide variety of climatic factors to consider. Additionally, the presence of other hosts and humans should not be underestimated.

One of these factors may be the reason behind the high number of positives for Windhoek. Positive results were obtained in alignment with the AHS season. The highest seasonal incidence occurs in the late summer and early autumn, between February and June (Coetzer & Gurthrie, 2004; Monaco *et al*., 2011; Gordon *et al*., 2013). There seems to be some high AHS incidence periods across the sites, since positive results were obtained from different sites on similar dates. Positive results were obtained in close proximity to the month (April) during which previous studies have found (via virus isolation) AHSV in *Culicoides*, specifically in Windhoek (Goffredo *et al*., 2015). Furthermore, the positive results for both 2013 and 2014 for the sites fall within the January to April timeframe, during which AHS cases have been reported in Namibia (Scacchia *et al*., 2015).

This study has shed some light onto the use of the vector for virus identification in areas at risk for AHS outbreaks. A technique was identified for using the *C. imicola* vector to draw information on AHSV presence and distribution. The study has shown the successful application of a RT-qPCR for the detection of AHSV in *C. imicola* pools both in the laboratory and in the field under different environmental circumstances. The importance of this application is far-reaching. Not only can it provide information about the *Culicoides* vectors in a specific area, but also the disease status of those vectors. Determining the presence of AHSV in a vector population allows for rapid action before an outbreak occurs in animals. Information regarding the spread and distribution of AHSV across various climatic conditions can be compared to the presence of AHSV in *Culicoides*. Since detection of AHSV is possible in a *C. imicola* complex, it is possible to apply this technique to other *Culicoides* species implicated as AHSV vectors or other arthropods (mosquitoes and ticks). The scope of possible vectors may be defined in such a manner. However, RT-qPCR is still being developed and improved (Scheffer, 2011).

It is important that further research be conducted on this application of RT-qPCR on vector species. It is recommended that the optimal pool size for the detection of a single infected *C. imicola* be further specified, in consideration of costs and available specimens. The inclusion of this application in AHSV epidemiological studies is suggested, based on the information obtained before an actual outbreak. Such a technique may be used to identify sites where no information is available on AHSV presence. It is suggested that this technique be applied to a wider array of vector species. Additionally, this application can be used before and after the

AHSV season, to draw information on persistence of the virus during low AHS incidence period (e.g. winter months).

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